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(54) Title: GENERATION OF XENOGENEIC ANTIBODIES

(57) Abstract

The subject invention provides non-human mammalian hosts characterized by inactivated endogenous Ig loci and functional human Ig loci for response to an immunogen to produce human antibodies or analogs thereof. The hosts are produced by multiple genetic modifications of embryonic cells in conjunction with breeding. Different strategies are employed for recombination of the human loci randomly or at analogous host loci. Chimeric and transgenic mammals, particularly mice, are provided, having stably integrated large, xenogeneic DNA segments. The segments are introduced by fusion with yeast spheroplasts comprising yeast artificial chromosomes (YACs) which include the xenogeneic DNA segments and a selective marker such as HPRT, and embryonic stem cells.

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GENERATION OF XENOGENEIC ANTIBODIES

CROSS-REFERENCE TO RELATED APPLICATIONS

This application is a continuation-in-part of Application Serial No. 07/919,297 filed July 24, 1992 which was a continuation-in-part of Application Serial No. 07/610,515 filed November 8, 1990 which was a continuation-in-part of Application Serial No. 07/466,008 filed January 12, 1990, the entire disclosures of which are all incorporated herein by reference.

INTRODUCTION

Technical Field

The field of this invention is the production of xenogeneic specific binding proteins in a viable mammalian host.

Background

The ability to produce transgenic animals has been revolutionized with the advent of the ability to culture murine embryonic stem cells, and to introduce genetic modifications in these cells for subsequent transmission to the mouse germline. Thus one has the opportunity to modify endogenous genes to produce animal strains capable of producing novel products by introduction of foreign genes into the host, particularly human genes to produce xenogeneic binding proteins. The expression of such genes in vivo in an animal model may provide for investigation of the function of the gene, the regulation of gene expression, its processing, response to various agents and the like. In addition, animals with new phenotypes,

including those that mimic a variety of diseases, may be produced. For example, there is interest in introducing a dominant mutation or complementing a recessive mutation. Depending on the particular gene, the difficulty of achieving the desired mutation will vary greatly. While some gene targets have proven to be relatively amenable to modification, other targets have proven to be extremely resistant to modification.

Because of the opportunity for generating transgenic animals, there is substantial interest in providing new procedures that increase the success of production of transgenic animals. Particularly, where one wishes to introduce large DNA fragments, encompassing hundreds of kilobases, there is substantial concern about the ability to introduce the large fragments in intact form into mammalian cells, the efficiency of integration, the functional capability of the gene(s) present on the fragment and transmission in the germline to the progeny. In addition, such procedures for introduction of large DNA fragments provide for determination of the function of large DNA fragments identified in the ongoing human genome project.

In particular, there is interest in producing xenogeneic specific binding proteins, for example human monoclonal antibodies, in small laboratory animals such as mice. Monoclonal antibodies find use in both diagnosis and therapy. Because of their ability to bind to a specific epitope, they can be uniquely used to identify molecules carrying that epitope or may be directed, by themselves or in conjunction with another moiety, to a specific site for diagnosis or therapy.

Monoclonal antibodies comprise heavy and light chains which join together to define a binding region for the epitope. Each of the chains is comprised of a variable region and a constant region. The constant region amino acid sequence is specific for a particular isotype of the antibody, as well as the host which produces the antibody.

Because of the relationship between the sequence of the constant region and the species from which the antibody is produced, the introduction of a xenogeneic antibody into the vascular system of the host can produce an immune response. Where the xenogeneic antibody is introduced repetitively, in the case of chronic diseases, it becomes impractical to administer the antibody, since it will be rapidly destroyed and may have an adverse effect. There have been, therefore, many efforts to provide a source of syngeneic or allogeneic antibodies. One technique has involved the use of recombinant DNA technology where the genes for the heavy and light chains from a host were identified and the regions encoding the constant region isolated. These regions were then joined to the variable region encoding portion of other immunoglobulin genes from another species directed to a specific epitope.

While the resulting chimeric partly xenogeneic antibody is substantially more useful than using a fully xenogeneic antibody, it still has a number of disadvantages. The identification, isolation and joining of the variable and constant regions requires substantial work. In addition, the joining of a constant region from one species to a variable region from another species may change the specificity and affinity of the variable regions, so as to lose the desired properties of the variable region. Also, there are framework and hypervariable sequences specific for a species in the variable region. These framework and hypervariable sequences may result in undesirable antigenic responses.

It would therefore be more desirable to produce allogeneic antibodies for administration to a host by immunizing the host with an immunogen of interest. For primates, particularly humans, this approach is not practical. The human antibodies which have been produced have been based on the adventitious presence of an available spleen, from a host which had been previously

immunized to the epitope of interest. While human peripheral blood lymphocytes may be employed for the production of monoclonal antibodies, these have not been particularly successful in fusions and have usually led only to IgM. Moreover, it is particularly difficult to generate a human antibody response against a human protein, a desired target in many therapeutic and diagnostic applications. There is, therefore, substantial interest in finding alternative routes to the production of allogeneic antibodies for humans.

Relevant Literature

Thomas and Capecchi (1987), Cell, 51:503-512 and Koller and Smithies (1989), Proc. Natl. Acad. Sci. USA, 86:8932-8935 describe inactivating the β2-microglobulin locus by homologous recombination in embryonic stem cells. Berman et al. (1988), EMBO J. 7:727-738 describe the human Ig VH locus. Burke, et al. (1987), Science, 236:806-812 describe yeast artificial chromosome vectors. See also, Garza et al. (1989), Science, 246:641-646 and Brownstein et al. (1989), Science, 244:1348-1351. Sakano, et al., describe a diversity segment of the immunoglobulin heavy chain genes in Sakano et al. (1981), Nature, 290:562-565. Tucker et al. (1981), Proc. Natl. Acad. Sci. USA, 78:7684-7688 describe the mouse IgA heavy chain gene sequence. Blankenstein and Kruwinkel (1987), Eur. J. Immunol., 17:1351-1357 describe the mouse variable heavy chain region. See also, Joyner et al. (1989), Nature, 338:153-155, Traver et al. (1989) Proc. Nat. Acad. Sci. USA 86:5898-5902, Pachnis et al. (1990), Proc. Nat. Acad. Sci. USA , 87:5109-5113 and PCT application PCT/US91/00245. Bruggemann et al., Proc. Nat. Acad. Sci. USA; 86:6709-6713 (1989); Behring Inst. Mitt. 87:21-24 (1990); Eur. J. Immunol. 21:1323-1326 (1991), describe monoclonal antibodies with human heavy chains. Albertsen et al., Proc. Nat. Acad. Sci. USA 87:4256-4260 (1990), describe the construction of a library of yeast artificial

chromosomes containing human DNA fragments. Yeast artificial chromosome vectors are described by Burke et al., Science 236:806-812 (1987). Pavan et al., Mol. and Cell. Biol. 10(8):4163-4169 (1990) describe the introduction of a neomycin resistance cassette into the human-derived insert of a yeast artificial chromosomes using homologous recombination and transfer into an embryonal carcinoma cell line using polyethylene glycol-mediated spheroplast fusion. Pachnis et al., Proc. Nat. Acad. Sci. USA 87:5109-5113 (1990), and Gnirke et al., EMBO Journal 10(7):1629-1634 (1991), describe the transfer of a yeast artificial chromosome carrying human DNA into mammalian cells. Eliceiri et al., Proc. Nat. Acad.USA 88:2179-2183 (1991), describe the expression in mouse cells of yeast artificial chromosomes containing human genes. Huxley et al., Genomics 9:742-750 (1991) describe the expression in mouse cells of yeast artificial chromosomes containing the human HPRT gene. Mortensen et al., Mol. and Cell. Biol. 12(5):2391-2395 (1992) describe the use of high concentrations of G418 to grow heterozygous embryonic stem cells for selection of homozygous mutationally altered cells. Yeast protoplast fusion with mouse fibroblasts is described by Traver et al., Proc. Nat. Acad. Sci. USA 86:5898-5902 (1989) and Pachnis et al., Proc. Nat. Acad. Sci. USA 87:5109-5113 (1990). Davies et al., Nucl. Acids Res. 20:2693-2698 (1992) describe targeted alterations in YACs. Zachau, Biol. Chem. 371:1-6 (1990) describes the human immunoglobulin light (kappa) (IgK) locus; Matsuda et al., Nature Genetics 3:88-94 (1993) and Shin et al., EMBO 10:3641-3645 (1991) describe the cloning of the human immunoglobulin heavy (IgH) locus in YACs.

SUMMARY OF THE INVENTION

Xenogeneic specific binding proteins are produced in a non-human viable host by immunization of the host with an appropriate immunogen.

A preferred non-human host is characterized by: (1) being incapable of producing endogenous immunoglobulin heavy chain; (2) being substantially incapable of producing endogenous immunoglobulin light chains; and (3) capable of producing xenogeneic immunoglobulin light and heavy chains to produce a xenogeneic immunoglobulin or immunoglobulin analog. Thus, the host may have an entire endogenous immunoglobulin locus substituted by a portion of, or an entire, xenogeneic immunoglobulin locus, or may have a xenogeneic immunoglobulin locus inserted into a chromosome of the host cell and an inactivated endogenous immunoglobulin region. These various alternatives will be achieved, at least in part, by employing homologous recombination for inactivation or replacement at the immunoglobulin loci for the heavy and light chains.

Additionally, novel methods are provided for introducing large segments of xenogeneic DNA of at least 100 kb, particularly human DNA, into host animals, particularly mice, by introducing a yeast artificial chromosome (YAC) containing a xenogeneic DNA segment of at least 100 kb, into an embryonic stem cell for integration into the genome of the stem cell, selection of stem cells comprising the integrated YAC by means of a marker present in the YAC, introduction of the YAC-containing ES cells into embryos and generation of chimeric mice from the embryos. The chimeric animals may be mated to provide animals that are heterozygous for the YAC. The heterozygous animals may be mated to generate progeny homozygous for the integrated YAC.

DESCRIPTION OF THE DRAWINGS

Figure 1 is a diagram of the inactivation vector for the mouse heavy chain J region, as described in Example I, infra.

Figure 2 is a diagram of the DNA restriction map for the plasmid pmHδJ and the targeted mouse heavy chain J genes, as described in Example II, infra.

Figure 3 is a flow cytometry plot of antibody staining for IgM allotypes in mouse strains, as described in Example II, infra.

Figure 4 is a flow cytometry histogram of antibody staining for IgM allotypes in mouse strains, as described in Example II, infra.

Figure 5 is a diagram of the inactivation vector for the mouse immunoglobulin kappa constant region genes, as described in Example III, infra.

Figure 6 is a diagram of the derivation of the plasmid pK.TK/Neo, as described in Example III, infra.

Figure 7 is a diagram of the restriction map of the light chain targeted locus, as described in Example III, infra.

Figure 8 is a diagram of the targeting vector for inactivation of the kappa light chain J and constant regions and design of the targeting experiment as described in Example IV, infra.

Figure 9 is a diagram of the construction of vectors for inactivating the kappa light chain J and constant regions as described in Example IV, infra.

Figure 10 is a diagram of the final deletion vectors for inactivation of the kappa light chain J and constant regions as described in Example IV, infra.

Figure 11 is an illustration of the Southern analysis of light chain J and constant region deleted cells as described in Example IV, infra.

Figure 12 A-E are photographs of the results of Southern blot analysis to characterize yHPRT and yeast genomic DNA integrated in ES clones as described in Example VI, infra (A = human repetitive Alu sequence; B,C = pBR322-specific sequences for the right (B) and left (C) YAC arms; D = yeast Ty repetitive sequence; E = yeast single copy gene LYS2. Shorter exposure times (12 hrs for II as compared to 48 hrs for I) of yHPRT probed with Alu and Ty sequences also are also shown. Positions of molecular weight markers are indicated. Schemes of right (a) and left (b) vector arms and the locations of pBR322-derived YAC vector fragments are shown (= telomere; = yeast-derived sequences; 0 = yeast centromere; = pBR322-derived sequences; = human insert; = EcoRI cloning site; H = HindIII sites).

Figure 13 A-D are photomicrographs of the results of in situ hybridization to detect integration of yHPRT and yeast genomic sequences in ES cell chromosomes as described in Example VI, infra (A, B =metaphase spreads from ESY 8-7 cells hybridized to biotinylated human genomic sequences and C = metaphase spreads or D = interphase nuclei from ESY 8-6 cells hybridized to biotinylated yeast repeated DNA sequences).

Figure 14 A, B, C demonstrates the stable retention of yHPRT during in vitro ES cell differentiation and transmission through the mouse germline, as described in Example VI, infra (A: a, b = embryoid bodies; and differentiated cell types:c = blood islands; d=contracting muscle; e= neuronal cells; f = neural tubules formed by ESY clones; B: Southern blot analysis of DNA extracted from differentiated ESY 5-2, 3-6, 8-5 and 8-6 (20 μg) and yHPRT in AB1380 (40 ng) using a = human Alu probe; b = yeast Ty sequences; C: Southern blot analysis of tail DNA (20 μg) from 2 agouti offspring (4-2 and 4-3) derived from ESY chimeric male 394/95-2 using a = human Alu and b = Ty sequences; shorter exposures (12 hr) of 8-6 and yHPRT probed with Ty are shown (II).

Figure 15 A and B are a photograph of an electrophoresis gel showing the expression of the human HPRT gene in various mouse tissues, as described in Example VI, infra (15 A = detection of human HPRT mRNA using reverse transcription-PCR in ES, ESY 3-1 and Hut 78 cells, spleen and liver from control mice or ESY 4-3 agouti offspring; 15 B = detection of mouse γ-interferon receptor mRNA by RT-PCR in samples from 15 A; M = size marker).

Figure 16 is a diagram of the human immunoglobulin heavy chain locus, and a human heavy chain replacement YAC vector, as described in Example VII, infra.

Figure 17 is a diagram of a mouse breeding scheme, as described in Example VIII, infra.

Figure 18 depicts the genotypes of some of the host animals produced by the methods of the invention.

DESCRIPTION OF SPECIFIC EMBODIMENTS

Novel transgenic non-human hosts, particularly mammalian hosts, usually murine, are provided, where the host is capable of mounting an immune response to an immunogen, where the response produces antibodies having xenogeneic, particularly primate, and more particularly human, constant and/or variable regions or such other effector peptide sequences of interest. By "transgenic" is meant an animal that contains a genetically engineered modification, particularly, as to this invention, the introduction of a human immunoglobulin gene, in all of its cells. The hosts are characterized by being capable of producing xenogeneic immunoglobulins or analogs thereof as a result of inactivation of the endogenous immunoglobulin subunit encoding loci and introduction of xenogeneic DNA, for example DNA encoding human immunoglobulin. The modifications may retain at least a portion of the xenogeneic constant regions which provide for assembly of the variable region binding site bonded at the C-terminus to a functional peptide. The functional peptide may take many forms or conformations and may serve as an enzyme, growth factor, binding protein, ligand, cytokine, effector protein, chelating proteins, etc. The antibodies may be of any isotype, e.g., IgA, D, E, G or M or subtypes within the isotype.

In a first strategy, as individual steps, the xenogeneic, e.g. human, heavy and light chain immunoglobulin genes are introduced into the host germ line (e.g. sperm or oocytes) and in separate steps the corresponding host genes are rendered non-functional by inactivation using homologous recombination. Human heavy and light chain immunoglobulin genes are reconstructed in an appropriate eukaryotic or prokaryotic microorganism and the resulting DNA fragments can be introduced into the appropriate host, for example into the pronuclei of fertilized mouse oocytes or embryonic stem cells.

Inactivation of the endogenous host immunoglobulin loci is achieved by targeted disruption of the appropriate loci by homologous recombination in the host cells, particularly embryonic stem cells or pronuclei of fertilized mouse oocytes. The targeted disruption can involve introduction of a lesion or deletion in the target locus, or deletion within the target locus accompanied by insertion into the locus, for example, insertion of a selectable marker. In the case of embryonic stem cells, chimeric animals are generated which are derived in part from the modified embryonic stem cells and are capable of transmitting the genetic modifications through the germ line. The mating of hosts with introduced human immunoglobulin loci to strains with inactivated endogenous loci will yield animals whose antibody production is purely xenogeneic, e.g. human.

In a second, alternative strategy, at least portions of the human heavy and light chain immunoglobulin loci are used to directly replace the corresponding endogenous immunoglobulin loci by homologous recombination in embryonic stem cells. This results in simultaneous inactivation and replacement of the endogenous immunoglobulin. This is followed by the generation of chimeric animals in which the embryonic stem cell-derived cells can contribute to the germ line.

These strategies are based on the known organization of the immunoglobulin chain loci in a number of animals, since the organization, relative location of exons encoding individual domains, and location of splice sites and transcriptional elements is understood to varying degrees. In the human, the immunoglobulin heavy chain (IgH_{bu}) locus is located on chromosome 14. In the 5' - 3' direction of transcription, the locus comprises a large cluster of variable region genes (V_H), the diversity (D) region genes, followed by the joining (J_H) region genes and the constant (C_H) gene cluster. The size of the locus is estimated to be about from 1,500 to about 2,500

kilobases (kb). During B-cell development, discontinuous gene segments from the germ line IgH locus are juxtaposed by means of a physical rearrangement of the DNA. In order for a functional heavy chain Ig polypeptide to be produced, three discontinuous DNA segments, from the V_H, D, and J_H regions must be joined in a specific sequential fashion; first D to J_H then V_H to DJ_H, generating the functional unit V_HDJ_H. Once a V_HDJ_H has been formed, specific heavy chains are produced following transcription of the Ig locus, utilizing as a template the specific $V_HDJ_HC_H$ unit comprising exons and introns.

There are two loci for immunoglobulin light chains (IgL), the kappa locus on human chromosome 2 and the lambda locus on human chromosome 22. The organization of the IgL loci is similar to that of the IgH locus, except that the D region is not present. Following IgH rearrangement, rearrangement of a light chain locus is similarly accomplished by V_L to J_L joining of the kappa or lambda chain. The sizes of the lambda and kappa loci are each approximately 1000 kb to 2000 kb. Expression of rearranged IgH and an Igκ or Igλ light chain in a particular B-cell allows for the generation of antibody molecules.

In order to isolate, clone and transfer the IgH_{hu} locus, a yeast artificial chromosome or "YAC" may be employed. A YAC carrying the xenogeneic DNA may be introduced into ES cells or oocytes by a variety of methods, including yeast spheroplast: ES cell fusion, microinjection and lipofection. The YAC will integrate randomly (i.e. non-homologously) into the host genome. If yeast spheroplast:ES cell fusion is employed to introduce a YAC carrying xenogeneic DNA into ES host cells, then two or more YACs in a single yeast host cell may be introduced simultaneously into the same host ES cell. The advantage of this approach is that multiple YACs each containing xenogeneic DNA, for example human heavy and light chain immunoglobulin loci, can be introduced

into a single chromosome in a host cell. This eliminates the need for breeding of animals containing individual human Ig genes in order to generate a host capable of producing fully human immunoglobulins. For example, a strain of yeast containing a single YAC is targeted with a vector such as pLUTO (described infra) to introduce a mammalian selectable marker such as HPRT, and a yeast selectable marker such as LYS2 into an arm of the YAC. Chromosomal DNA from the targeted strain is then used to transform a second, usually haploid, lys2 mutant yeast strain containing a second, different YAC. Lys+ colonies are then analyzed by pulsed-field gel electrophoresis (PFGE) to identify clones harboring the two YACs and to confirm that they are unaltered in size. Additional YACs with different selectable markers, for example ADE2 (if the host is an ade2 mutant), can subsequently be added by transformation. Alternatively, a YAC-containing strain of yeast is targeted with a vector such as pLUTO to introduce a mammalian selectable marker (e.g. HPRT), as above, and then mated to a second YAC-containing strain of opposite mating type. The presence of the two YACs is then confirmed in the diploid yeast cells as described above. The diploid yeast strain is used directly for fusion or put through meiosis and ascosporogenesis (sporulation) using standard procedures. The meiotic products are then screened to identify a haploid clone containing the two YACs. With either approach described above, the second YAC can be targeted with HPRT or another selectable marker prior to introduction of the first YAC. Also, if each YAC contains a different yeast selectable marker, maintenance of both YACS during strain propagation may be genetically selected. Fusion with ES cells is then carried out in the same manner as with yeast cells containing a single YAC. Because many yeast chromosomes may integrate along with the YAC, it is expected that a substantial portion of ES clones expressing the mammalian selectable marker present in one YAC (e.g. HAT^R clones

if the YAC marker is HPRT, and the ES cells are HPRT-), will have integrated both YACs. Methods such as Southern analysis and/or PCR may be used to identify such clones, and Southern analysis employing pulsed-field gel electrophoresis used to characterize the extent of YAC integration.

The entire IgH_{Hu} locus can be contained within one or a few YAC clones along with a mammalian marker such as Neo, HPRT, GPT, β-gal, etc. The same is true for the Ig light chain loci. Reconstitution of intact germ line Ig loci by homologous recombination between YACs with overlapping regions of homology can be achieved in yeast.. In this manner, the isolation of DNA fragments encoding the human Ig chain is obtained. Alternatively, one can directly clone an intact germline locus in a single YAC.

In order to obtain a broad spectrum of high affinity antibodies, it is not necessary that one include the entire V region. Various V region gene families are interspersed within the V region cluster in humans. Thus, by obtaining a subset of the known V region genes of the human heavy and light chain Ig loci (Berman et al., EMBO J. (1988) 7:727-738) rather than the entire complement of V regions, the transgenic host may be immunized and be capable of mounting a strong immune response and provide high affinity antibodies. In this manner, relatively small DNA fragments of the chromosome may be employed. For example, a reported 670 kb fragment of the Ig_{Hu} locus is contained on a NotI-NotI restriction fragment, which would serve to provide a variety of V regions (Berman et al., supra). Increased diversity is also provided by recombination with the various D and J regions and somatic mutation.

To render the host immunoglobulin loci non-functional, homologous recombination may be employed, where DNA is introduced at the endogenous host immunoglobulin heavy chain and light chain loci which inhibits the production of endogenous immunoglobulin.

Because there are two heavy chain alleles and two light chain loci, kappa and lambda, each with two alleles, although one may choose to ignore the lambda loci, there will have to be multiple transformations which result in inactivation of each of the alleles. Homologous recombination may be employed to functionally inactivate each of the loci, by introduction of the homologous DNA via a construct that can disrupt or delete the target locus into embryonic stem cells, followed by introduction of the modified cells into recipient blastocysts. Subsequent breeding allows for germ- line transmission of the inactivated locus. One can therefore choose to breed heterozygous offspring and select for homozygous offspring from the heterozygous parents.

In the second, alternative strategy described above, the number of steps may be reduced by providing at least a fragment of the human immunoglobulin locus within the construct used for homologous recombination with the analogous endogenous immunoglobulin, so that the human locus is substituted for at least a part of the host immunoglobulin locus, with resulting inactivation of the host immunoglobulin subunit locus. Of particular interest is the use of transformation for a single inactivation, followed by breeding of the heterozygous offspring to produce a homozygous offspring. Where the human locus is employed for substitution or insertion into the host locus for inactivation, the number of transformations may be limited to three transformations and as already indicated, one may choose to ignore the less used locus and limit the transformations to two transformations. Alternatively, one may choose to provide for inactivation as a separate step for each locus, employing embryonic stem cells from offspring which have previously had one or more loci inactivated. In the event that only transformation is used and the human locus is integrated into the host genome in random fashion, a total of eight or more transformations may be required.

For inactivation, any lesion in the target locus resulting in the prevention of expression of an immunoglobulin subunit of that locus may be employed. Thus, the lesion may be in a region comprising enhancers, e.g., a 5′ or 3′ enhancer, or intron, in the V, J or C regions, and with the heavy chain, the opportunity exists in the D region, or combinations thereof. The important factor is that Ig germ line gene rearrangement is inhibited, or a functional message encoding the enodgenous immunoglobulin cannot be produced, either due to failure of transcription, failure of processing of the message, or the like. Such a lesion may take the form of a deletion in the target gene, an insertion of a foreign gene, a combination of an insertion and deletion, or a replacement using xenogeneic sequences with or without introduction of a deletion in the endogenous gene.

Preferably, when one is interested in inactivating the immunoglobulin subunit locus, the lesion will be introduced into one or more of the exons contained in the immunoglobulin subunit locus, for example in the constant or J region of the locus. Thus, one produces a targeting construct which lacks functional exons in this region and may comprise the sequences adjacent to and upstream and/or downstream from the J and/or C region or comprises all or part of the region with an inactivating insertion in the J or C exons. The insertion may be 50 bp or more, where such an insertion results in disruption of formation of a functional mRNA. Desirably, usually at least about 75% of the exon sequence, preferably at least about 90% of the exon sequence, is deleted.

Desirably, a marker gene is used in the targeting construct to replace the deleted sequences. Various markers may be employed, particularly those which allow for positive selection. Of particular interest is the use of G418 resistance, resulting from expression of the gene for neomycin phosphotransferase ("neo").

In the targeting construct, upstream and/or downstream from the target gene, may be a gene which provides for identification of whether a homologous double crossover has occurred (negative selection). For this purpose, the Herpes simplex virus thymidine kinase gene may be employed, since cells expressing the thymidine kinase gene may be killed by the use of nucleoside analogs such as acyclovir or gancyclovir, by their cytotoxic effects on cells that contain a functional HSV-tk (Mansour et al., Nature 336:348-352 (1988)). The absence of sensitivity to these nucleoside analogs indicates the absence of the HSV-thymidine kinase gene and, therefore, where homologous recombination has occurred, that a double crossover has also occurred.

While the presence of the marker gene in the genome will indicate that integration has occurred, it will still be necessary to determine whether homologous integration has occurred. This can be achieved in a number of ways. For the most part, DNA analysis by Southern blot hybridization will be employed to establish the location of the integration. By employing probes for the insert and the sequences at the 5′ and 3′ regions flanking the region where homologous integration would occur, one can demonstrate that homologous targeting has occurred.

PCR may also be used with advantage in detecting the presence of homologous recombination. PCR primers may be used which are complementary to a sequence within the targeting construct and complementary to a sequence outside the construct and at the target locus. In this way, one can only obtain DNA molecules having both the primers present in the complementary strands if homologous recombination has occurred. By demonstrating the expected size fragments, e.g. using Southern blot analysis, the occurrence of homologous recombination is supported.

The targeting construct may further include a replication system which is functional in the host cell. For the most part, these replication systems will involve

viral replication systems, such as Simian virus 40, Epstein-Barr virus, polyoma virus, papilloma virus, and the like. Various transcriptional initiation systems may be employed, either from viruses or from mammalian genes, such as SV40, metallathionein-I and II genes, β-actin gene, adenovirus early and late genes, phosphoglycerate kinase gene, RNA polymerase II gene, or the like. In addition to promoters, wild-type enhancers may be employed to further enhance the expression of the marker gene.

In preparing the targeting constructs for homologous recombination, a replication system for procaryotes, particularly E. coli, may be included for preparing the targeting construct, subcloning after each manipulation, analysis such as restriction mapping or sequencing, expansion and isolation of the desired sequence. In the case of the replacement strategy, where the xenogeneic DNA insert is large, generally exceeding about 50 kbp, usually exceeding 100 kbp, and usually not more than about 1000 kbp, a yeast artificial chromosome (YAC) may be used for cloning of the targeting construct.

Once a targeting construct has been prepared and any undesirable sequences removed, e.g., procaryotic sequences, the construct may now be introduced into the target cell, for example an ES cell. Any convenient technique for introducing the DNA into the target cells may be employed. Techniques include protoplast fusion, e.g. yeast spheroplast:cell fusion, lipofection, electroporation, calcium phosphate-mediated DNA transfer or direct microinjection.

After transformation or transfection of the target cells, target cells may be selected by means of positive and/or negative markers, as previously indicated, neomycin resistance and acyclovir or gancyclovir resistance. Those cells which show the desired phenotype may then be further analyzed by restriction analysis, electrophoresis, Southern analysis, PCR, or the like. By identifying fragments which show the presence of the lesion(s) at the

target locus, one can identify cells in which homologous recombination has occurred to inactivate a copy of the target locus.

The above described process may be performed first to inactivate a heavy chain locus in an embryonic stem cell whereby the cells are microinjected into host blastocysts which develop into a chimeric animal. The chimeric animals are bred to obtain heterozygous hosts. Then, by breeding of the heterozygous hosts, a homozygous host may be obtained or embryonic stem cells may be isolated and transformed to inactivate the second IgH locus, and the process repeated until all the desired loci have been inactivated. Alternatively, the light chain locus may be the first to be inactivated. For complete elimination of the ability to produce light chain immunoglobulin, it is desirable to inactivate both the lambda and the kappa light chain immunoglobulin loci. At any stage, the xenogeneic loci may be introduced.

As already indicated, the target locus may be substituted with the analogous xenogeneic locus. In this way, the xenogeneic locus will be placed substantially in the same region as the analogous host locus, so that any regulation associated with the position of the locus will be substantially the same for the xenogeneic immunoglobulin locus. For example, by isolating the variable region of the human IgH locus (including V, D, and J sequences), or portion thereof, and flanking the human locus with sequences from the murine locus, preferably sequences separated by at least about 5 kbp, in the host locus, preferably at least about 10 kbp in the host locus, one may insert the human fragment into this region in a recombinational event(s), substituting the human immunoglobulin locus for the endogenous variable region of the host immunoglobulin locus. In this manner, one may disrupt the ability of the host to produce an endogenous immunoglobulin subunit, while allowing for the promoter of the human immunoglobulin locus to be activated

by the host enhancer and regulated by the regulatory system of the host.

In order to provide for the production of xenogeneic binding proteins in a host, it is necessary that the host be competent to provide the necessary enzymes and other factors involved with the production of antibodies, while lacking competent endogenous genes for the expression of heavy and light subunits of immunoglobulins. Thus, those enzymes and other factors associated with germ line rearrangement, splicing, somatic mutation, and the like will be functional in the host. What will be lacking is a functional natural region comprising the various exons associated with the production of endogenous immunoglobulin.

The integration of introduced xenogeneic DNA may be random or homologous depending on the particular strategy to be employed. Thus, by using transformation, using repetitive steps or in combination with breeding, transgenic animals may be obtained which are able to produce xenogeneic binding proteins in the substantial absence of light or heavy endogenous immunoglobulin. By transformation is intended any technique for introducing DNA into a viable cell, such as conjugation, PEG-mediated cell fusion, transformation, transfection, transduction, electroporation, lipofection, biolistics, or the like.

Once the xenogeneic loci, have been introduced into the host genome, either by homologous recombination or random integration, and host animals have been produced with the endogenous immunoglobulin loci inactivated by appropriate breeding of the various transgenic animals or animals derived from chimeric animals, one can produce a host which lacks the native capability to produce endogenous immunoglobulin, but has the capacity to produce xenogeneic immunoglobulins with at least a significant portion of the repertoire of the xenogeneic source.

The functional inactivation of the two copies of each of the three host Ig loci (heavy, kappa and lambda), where

the host then contains the human IgH and the human Ig kappa and/or lambda loci would allow for the production of purely human antibody molecules without the production of host or host/human chimeric antibodies. Such a host strain, by immunization with specific antigens, would respond by the production of murine B-cells producing specific human antibodies, which B-cells could be fused with murine myeloma cells or be immortalized in any other manner for the continuous stable production of human monoclonal antibodies. Methods are well known in the art for obtaining continuous stable production of monoclonal antibodies.

The subject methodology and strategies need not be limited to producing complete immunoglobulins, but provides the opportunity to provide for regions joined to a portion of the constant region, e.g., C_{H1}, C_{H2}, C_{H3}, or C_{H4}, or combination thereof. Alternatively, one or more of the exons of the C_H and C_κ or C_λ regions may be replaced or joined to a sequence encoding a different protein, such as an enzyme, e.g., plasminogen activator, superoxide dismutase, etc.; toxin, e.g., ricin, abrin, diphtheria toxin, etc.; growth factor; cytotoxic agent, e.g., TNF; receptor ligand, or the like. See, for example, WO 89/07142; WO 89/09344; and WO 88/03559. By inserting the protein of interest into a constant region exon and providing for splicing of the variable region to the modified constant region exon, the resulting binding protein may have a different C-terminal region from the immunoglobulin. By providing for a stop sequence with the inserted gene, the protein product will have the inserted protein as the C-terminal region. If desired, the constant region may be entirely substituted by the other protein, by providing for a construct with the appropriate splice sites for joining the variable region to the other protein.

The B-cells from the transgenic host producing immunoglobulin or immunoglobulin analog may be used for

fusion to a murine myeloid cell to produce hybridomas or immortalized by other conventional process, e.g., transfection with oncogenes. These immortalized cells may then be grown in continuous culture or introduced into the peritoneum of a compatible host for production of ascites.

The subject invention provides for the production of polyclonal human anti-serum or human monoclonal antibodies or antibody analogs. Where the mammalian host has been immunized with an immunogen, the resulting human antibodies may be isolated from other proteins by using an affinity column, having an Fc binding moiety, such as protein A, or the like.

The invention includes the following embodiments of non-human hosts (see also Figure 18):

I. Animals heterozygous for an inactive endogenous light chain immunoglobulin gene (homozygous animals are obtained by interbreeding);

II. Animals heterozygous for an inactive endogenous heavy chain immunoglobulin gene (homozygous animals are obtained by interbreeding);

III. Animals homozygous for functional endogenous light and heavy chain immunoglobulin genes and hemizygous for (i.e. containing one copy of) foreign, preferably human, heavy chain immunoglobulin genes (homozygous animals are obtained by interbreeding);

IV. Animals homozygous for functional endogenous light and heavy chain immunoglobulin genes and hemizygous for foreign, preferably human, light chain immunoglobulin genes (homozygous animals are obtained by interbreeding);

V. Animals heterozygous for inactive endogenous heavy and light chain immunoglobulin genes obtained by crossbreeding animals of category I with animals from category II (homozygous animals are obtained by interbreeding);

VI. Animals heterozygous for inactive endogenous heavy and light chain immunoglobulin genes and hemizygous for foreign, preferably human, heavy chain immunoglobulin genes obtained by crossbreeding animals of category III with animals from category V (animals homozygous for the inactive endogenous loci and homo- or hemizygous for the foreign gene are obtained by interbreeding);

VII. Animals heterozygous for inactive endogenous heavy and light chain immunoglobulin genes and hemizygous for foreign, preferably human, light chain immunoglobulin genes obtained by crossbreeding animals of category IV with animals from category V (animals homozygous for the inactive endogenous loci and homo- or hemizygous for the foreign gene are obtained by interbreeding);

VIII. Animals homozygous or heterozygous for inactive endogenous heavy and light chain immunoglobulin genes and hemizygous for foreign, preferably human, light and heavy chain immunoglobulin genes, obtained by crossbreeding animals of category VI and VII (animals homozygous for the inactive endogenous loci and homo- or hemizygous for the foreign gene are obtained by interbreeding);

In a preferred embodiment, the homozygous animals of category VIII are used to produce human antibodies.

IX. Animals homozygous for functional endogenous heavy and light chain immunoglobulin genes and hemizygous for foreign, preferably human, heavy and light chain immunoglobulin genes, obtained by crossbreeding animals of category III and IV (homozygous animals are obtained by interbreeding);

X. Animals heterozygous for an inactive endogenous heavy chain immunoglobulin gene and hemizygous for foreign, preferably human, heavy and light chain immunoglobulin genes, obtained by crossbreeding animals of category II and IX (animals homozygous for the inactive endogenous loci and homo- or hemizygous for the foreign gene are obtained by interbreeding).

XI. Animals heterozygous for an inactive endogenous light chain immunoglobulin gene and hemizygous for foreign, preferably human, heavy and light chain immunoglobulin genes, obtained by crossbreeding animals of category I and IX (animals homozygous for the inactive endogenous loci and homo- or hemizygous for the foreign gene are obtained by interbreeding).

The invention also provides a method for introducing large continuous, xenogeneic DNA sequences into a non-human, e.g. mammalian, host. Usually, the sequences will be at least 100 kb, more usually at least about 200 kb, generally ranging from about 200 to 1000 kb. Thus, one may wish to transfer a locus of interest, such as the immunoglobulin locus, T-cell receptor locus, major histocompatibility locus; regions of an xenogeneic chromosome, which may include one or more genes of interest, which may or may not have been characterized, such as the Low Density Lipoprotein (LDL) receptor, Apolipoprotein (Apo) B, Apo E, cystic fibrosis transmembrane conductor regulator, dystrophin, or regions of xenogeneic chromosomes that may be involved in partial chromosome trisomy (e.g. chromosomes 21, 7 and 10); and viruses. The DNA may comprise wild type or defective genes for studying a variety of diseases by creating dominant mutations or complementing recessive mutations, for example the LDL receptor and Apo B genes can be introduced for the study of hypercholesterolemia, hyperlipoproteinemia and atherosclerosis, Factor VIII or IX can be introduced for hemophilia, cystic fibrosis transmembrane conductance regulator can be introduced for cystic fibrosis and the dystrophin gene for muscular dystrophy. The xenogeneic DNA to be introduced using a YAC is from a mammalian source, particularly primates, more particularly human, other vertebrates or invertebrates and the like. One can thus impart numerous novel capabilities to the host, create genetic responses related to the xenogeneic source of the DNA, provide for

the production of antibodies, provide for specific combinations of transcription factors, provide for metabolic systems, introduce dominant mutations or complement recessive mutations. The xenogeneic DNA may be modified when present in a YAC. Because homologous recombination is efficient in yeast, giving a high ratio of site-specific integration of homologous DNA, where the homologous DNA flanks other DNA of interest, one is able to modify the xenogeneic DNA before introduction into an ES cell. In this way, one can introduce defective genes into the host which express defective proteins to mimic diseased states of the xenogeneic host, to study various mechanisms of the interaction of defective proteins with other xenogeneic proteins or endogenous proteins, or to study genes or gene systems.

In general, to transfer large DNA segments, as described in detail herein, YACs are employed which comprise a yeast centromere, an origin of replication and telomeres bounding the DNA of interest. Various centromeres or telomeres may be used, particularly the centromeres from yeast chromosomes 4 and 5. The YAC has a marker which allows for selection or screening of cells into which the YAC becomes integrated. Not all markers allow for efficient selection. Particularly, the HPRT gene, more particularly human HPRT, is found to permit efficient selection of HPRT-deficient ES cells carrying the YAC. Other known selectable or screenable markers include hygromycin, neomycin, β-gal, and GPT. The ES cell may be derived from any non-human host, from which ES cells are available, and can be expanded in culture, which remain viable and functional, for which a marker for selection exists, and where the ES cell can be introduced into an embryo and can repopulate the host, including the germline. For the most part this capability has been established with rodents, e.g. mice and rats, and to a lesser extent with guinea pigs. Mice have been used for the production of antibodies or B-lymphocytes for

immortalization for the production of antibodies. Because mice are easy to handle, can be produced in large quantities, and are known to have an extensive immune repertoire, mice will usually be the animal of choice. As other species of ES cells become available, these may also be employed in accordance with the subject invention. Of particular interest will be small laboratory animals, or domestic animals particularly rodents, including mice, rats, rabbits, cows, pigs, hamsters, horses, dogs, sheep and guinea pigs, or birds such as chickens, turkeys, etc. The ES cells may have one or more mutations, for example lacking a particular activity. Of particular interest in this invention are ES cells that are deficient in HPRT. In addition, fertilized eggs of certain species may find use in accordance with the invention.

The YAC may be obtained by screening existing human YAC libraries such as those available from the Centre d'Etude du Polymorphisme Human (C.E.P.H.), Paris, France, and Washington University, St. Louis, MO, using standard procedures. Alternatively, the YAC is readily prepared as described in detail herein, by joining the yeast flanking segments comprising one arm with a centromere and telomere and another with a telomere together with the DNA of interest. Usually there will also be one or more markers present that allow for selection in the yeast host cells. For yeast selection, of particular interest are markers which complement mutations of the yeast host, such as genes involved in the production of amino acids, purines or pyrimidines, URA3, TRP1, LYS2, ADE2 on the YAC to complement ura3, trp1, lys2 and Ade2 mutations in the host. By providing for complementation, for the most part only yeast cells carrying the entire YAC will be able to survive in a selective medium. In addition to genetic verification that both YAC arms have been retained, it is desirable to confirm the integrity of the YAC using a method such as pulsed-field gel electrophoresis.

Those yeast hosts carrying the YAC may then be used as a source of the YAC for introduction into the ES cell. Transfer of the YAC is efficiently achieved by preparing yeast spheroplasts in accordance with conventional ways. By degrading the outer wall, under mild conditions, in an isotonic medium, spheroplasts are produced in high yield. Exponentially growing ES cells are protease-treated, e.g. trypsinized, and combined with the spheroplasts. Conveniently, a pellet of yeast spheroplasts can be prepared and the ES cells are spun with the pellet and exposed to a fusogenic agent such as PEG for 1-2 minutes. The cells are then resuspended and incubated in appropriate serrum-free medium. The cells are then plated onto feeder cells, followed by selection in accordance with the selective marker. For the HPRT gene, HAT medium may be employed for selection. Surviving fusion colonies are then, picked, expanded and analyzed. Analysis may be performed by restriction enzyme analysis, combined with Southern blotting or pulsed-field gel electrophoresis, or by the polymerase chain reaction (PCR), employing appropriate primers, at least one of which is complementary to the DNA insert, and probing with repetitive sequences present in the xenogeneic DNA, such as Alu, for detection of human DNA sequences. Ty, Y', rDNA, delta sequences are used to probe for for yeast sequences. Probes for YAC ends are used to confirm integrity of the YAC. Those cells that demonstrate the intact or substantially intact YAC DNA integrated into the host genome are then used in the next steps. In some clones, only a portion or little or none of the yeast DNA becomes integrated into the mouse genome. The integrated yeast DNA ranges from more than about 90% of the original yeast genome to less than about 10% .

In a preferred embodiment, efficient production of transgenic non-human hosts is provided using a process which integrates large, at least 100 kb, xenogeneic DNA fragments, in substantially intact form, into a host

embryonic stem (ES) cell or fertilized egg (zygote). The introduction of the xenogeneic DNA is efficiently achieved by fusion of the ES cell with yeast spheroplasts that contain YACs carrying the 100 kb DNA and a selectable marker, under conditions allowing for integration of the YAC DNA containing the marker into the ES cell genome, or by transfection of a purified YAC into ES cells. ES cells comprising the YAC integrated into the genome are then selected by means of the marker, which is functional in the ES cell. For example, the hypoxanthine phosphoribosyl transferase (HPRT) gene may be used as a marker in HPRT deficient (HPRT-) ES cells. For producing animals from embryonic stem cells, after transformation, the cells may be plated onto a feeder layer in an appropriate medium, e.g. fetal bovine serum enhanced DMEM. The ES cell may have a single targeted locus (heterozygous), or may be manipulated by the process of homogenotization to have both loci targeted (homozygous). The process of homogenotization (formation of homozygotes) uses selective pressure to grow out those cells which have the gene targeting event on both chromosomes. Cells containing the two targeted alleles may be detected by employing a selective medium and after sufficient time for colonies to grow, colonies may be picked and analyzed for the occurrence of integration or homologous recombination. As described previously, the PCR may be used, with primers within or outside of the construct sequence, but at the target locus.

Those colonies which show homologous recombination may then be used for embryo manipulation and blastocyst injection. The selected ES cells are then introduced into embryos, by microinjection or other means, into the appropriate host. For example, murine blastocyts may be obtained from female animals by flushing the uterus 3.5 days after ovulation. The modified ES cells are then trypsinized and at least 1 and up to 15 cells may be injected into the blastocoel of the blastocyst. After

injection, at least 1 and no more than about 10 of the blastocysts are returned to each uterine horn of pseudo-pregnant females. The females proceed to term and the resulting chimeric animals can be analyzed for the presence of the YAC in their somatic cells. By "chimeric" is meant an animal that carries cells derived from more than one source, e.g. from the host and another animal. For example, in the present invention a chimeric murine animal contains a genetically engineered modification, particularly a human gene, in some of its cells, e.g. in cells that develop from the modified embryonic stem cells. The presence of the integrated YAC in chimeric hosts that are generated is then analyzed. The chimeric hosts are evaluated for germline transmission of the ES cell genome by mating, for example chimeric mice are mated with C57BL/6J mice. Chimeric hosts may be bred with non-chimeric hosts, either syngeneic or allogeneic, to screen for chimeras that carry the YAC in their germ cells. Offspring that are heterozygous for the genetic modification are then interbred to produce progeny that are homozygous for the modification, stably transmitting the functioning YAC construct to their progeny.

The method of the invention for introduction of large xenogeneic DNA segments into a non-human host, particularly a rodent and usually a murine animal, provides for stable integration of the DNA. Genes in the inserted DNA are found to be functional and the resulting chimeric hosts are able to provide for germline transmission of the integrated DNA. After breeding of the chimeric host, transgenic heterozygous hosts are produced and are mated to produce a homozygous animal that may be used for a wide variety of purposes, including production of products, such as binding proteins, for example immunoglobulins, for screening of various drugs, for gene therapy, for example to complement for recessive genetic disorders, to study various diseases, to study

the function and regulation of poorly mapped large DNA fragments.

The following examples are offered by way of illustration and not by way of limitation.

EXPERIMENTAL

EXAMPLE I

I. Inactivation of the mouse heavy chain J (J_H) genes

A. Construction of the targeting inactivation vector

A 6.4 kb EcoRI fragment, containing the mouse heavy chain J genes and flanking sequences, is cloned from a Balb/c mouse embryo genomic library using the probes described in Sakano et al. (1981), Nature 290:562-565. This fragment (mDJ) is inserted into EcoRI-digested pUC19 plasmid (pmDJ). A 2.9 Kb fragment, containing the 4 J genes, is deleted by XhoI-ScaI digestion (pmDδJNeo, see Figure 1). An 1150 bp XhoI-BamHI fragment, containing a neomycin-resistance gene driven by the Herpes simplex virus thymidine kinase gene (HSV-tk) promoter and a polyoma enhancer is isolated from pMClNeo (Thomas and Capecchi (1987), Cell, 51, 503-512). A synthetic adaptor is added onto this fragment to convert the BamHI end into a ScaI end and the resulting fragment is joined to the XhoI-ScaI pmDδJ to form the inactivation vector (pmDδJ.Neo) in which the 5′ to 3′ orientation of the neomycin and the heavy chain promoters is identical. This plasmid is linearized by NdeI digestion before transfection to ES cells. The sequences driving the homologous recombination event are 3 kb and 0.5 kb fragments, located 5′ and 3′ to the neomycin gene, respectively.

B. Culturing, Electroporation and Selection of ES Cells

The ES cell line E14TG2a (Hooper et al. (1987), Nature, 326:292-295) is cultured on mitomycin treated primary embryonic fibroblast-feeder layers essentially

as described (Doetschman et al. (1985), J. Embryol. Exp. Morphol. 87:27-45). The embryonic fibroblasts are prepared from embryos from C57BL/6 females that are mated 14 to 17 days earlier with a male homozygous for a neomycin transgene (Gossler et al. (1986), PNAS 83:9065-9069). These cells are capable of growth in media containing G418. Electroporation conditions are described by (Boggs et al. (1986), Ex. Hematol. (NY) 149:988-994). ES cells are trypsinized, resuspended in culture media at a concentration of $4x10^7$/ml and electroporated in the presence of the targeting DNA construct at a concentration of 12nM in the first experiment and 5nM DNA in the second. A voltage of 300 V with a capacitance of 150-250 μF is found optimal with an electroporation cell of 5 mm length and 100 mm^2 cross-section. $5x10^6$ electroporated cells are plated onto mitomycin-treated fibroblasts in 100 mm dishes in the presence of Dulbecco's modified Eagle's media (DMEM) supplemented with 15% fetal bovine serum (FBS) and 0.1 mM 2-mercaptoethanol. The media is replaced 24 hrs after electroporation with media containing 200 μg/ml G418.

ES colonies resulting 10-14 days after electroporation are picked with drawn out capillary pipettes for analysis using PCR. Half of each picked colony is saved in 24-well plates already seeded with mitomycin-treated feeder cells. The other halves, combined in pools of 3-4, are transferred to Eppendorf tubes containing approximately 0.5 ml of PBS and analyzed for homologous recombination by PCR. Conditions for PCR reactions are essentially as described (Kim and Smithies (1988), Nucleic Acids Res. 16:8887-8893). After pelleting, the ES cells are resuspended in 5 μl of PBS and are lysed by the addition of 55 μl of H_2O to each tube. DNAses are inactivated by heating each tube at 95°C for 10 min. After treatment with proteinase K at 55°C for 30 min, 30 μl of each lysate is transferred to a tube containing 20 μl of a reaction mixture including PCR

buffer: 1.5 μg of each primer, 3U of Taq polymerase, 10% DMSO, and dNTPs, each at 0.2 mM. The PCR expansion employs 55 cycles using a thermocycler with 65 seconds melt at 92°C and a 10 min annealing and extension time at 65°C. The two priming oligonucleotides are TGGCGGACCGCTATCCCCCAGGAC and TAGCCTGGGTCCCTCCTTAC, which correspond respectively to a region 650 bases 3′ of the start codon of the neomycin gene and sequences located in the mouse heavy chain gene, 1100 bases 3′ of the insertion site. 20 μl of the reaction mix is electrophoresed on agarose gels and transferred to nylon membranes (Zeta Bind). Filters are probed with a ^{32}P-labelled fragment of the 991 bp XbaI fragment of the J-C region.

EXAMPLE II

II. Deletion of the mouse Ig heavy chain J (J_H) genes in ES cells

A. Construction of the replacement targeting vector

A 6.1-Kb EcoRI fragment, containing the mouse immunoglobulin heavy chain J region genes and flanking sequences, cloned from a BALB/c mouse embryo genomic library and inserted into pUC18 (pJ_H), was digested with XhoI and NaeI to delete an about 2.3 kb fragment containing the four J genes (see Figure 2A). An about 1.1 kb XhoI-BamHI fragment, blunted at the BamHI site, containing a neomycin resistance gene driven by the Herpes simplex virus thymidine kinase gene (HSV-tk) promoter and polyoma enhancer was isolated from pMC1Neo (Thomas and Capecchi (1987), Cell, 51, 503-512). This fragment was inserted into the XhoI-NaeI deleted pJH to form the deletion vector (pmHδJ, see Figure 2B), in which the transcriptional orientation of the neomycin and the heavy chain genes is the same. This plasmid was linearized by NdeI digestion before transfection to ES cells. The sequences driving the homologous recombination event are

about 2.8 kb and about 1.1 kb fragments, located 5′ and 3′ to the neomycin gene, respectively.

B. Culturing, Electroporation, and Selection of ES cells

The ES cell line E14TG2a (Koller and Smithies (1989), PNAS USA, 86:8932-8935) was cultured on mitomycin C-treated embryonic fibroblast feeder layers as described (Koller and Smithies (1989), PNAS USA, 86:8932-8935). ES cells were trypsinized, resuspended in HBS buffer (pH 7.05; 137 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 0.7 mM Na_2HPO_4, 21 mM HEPES pH 7.1) at a concentration of $2x10^7$/ml and electroporated in the presence of 50 μg/ml of the linearized inactivation vector. Electroporation was carried out with a BioRad Gene Pulser using 240 volts and 500 μF capacitance. $5x10^6$ electroporated cells were plated onto mitomycin C-treated fibroblasts in 100 mm dishes in the presence of Dulbecco's modified Eagle's media (DMEM) supplemented with 15% fetal bovine serum and 0.1 mM 2-mercaptoethanol. The media was replaced 24 hr after electroporation with media containing 200 μg/ml G418. G418-resistant ES colonies resulting from growth 12-14 days after electroporation were picked with drawn out capillary pipettes for analysis using the polymerase chain reaction (PCR). Half of each picked colony was transferred to an individual well of a 24-well plate, already seeded with mitomycin C-treated feeder cells. The other halves, combined in pools of four, were transferred to Eppendorf tubes containing 0.3 ml of PBS and cell lysates were prepared for PCR analysis as described by Joyner et al (1989) Nature, 338:153-155. The PCR reaction included 5-20 μl of the cell lysate, 1 μM of each primer, 1.5 U of Taq polymerase and 200 μM of dNTPs. The PCR amplification employed 45 cycles using a thermal cycler (Perkin-Elmer Cetus), with 1 min. melt at 94°C, 2 min. annealing at 55°C, and 3 min. extension at 72°C. The two priming oligonucleotides are ACGGTATCGCCGCTCCCGAT and AGTCACTGTAAAGACTTCGGGTA, which

correspond respectively to about 120 bases 5' of the BamHI site of the neomycin gene, and to the sequences located in the mouse heavy chain gene, about 160 bases 3' of the insertion site. Successful homologous recombination gives rise to an about 1.4 kb fragment. 20 μl of the reaction mixture is electrophoresed on 1% agarose gels, stained with ethidium bromide and transferred to nylon membranes (Gene Screen). Filters were probed with a ^{32}P-labelled EcoRI-PstI about 1.4 kb fragment located in the mouse heavy chain, 3' of the insertion site (see Figure 2). For further analysis, genomic DNA was prepared from ES cells, digested with restriction enzymes as recommended by the manufacturers, and fragment were separated on 1% agarose gels. DNA was transferred to nylon membranes (Gene Screen) and probed with the ^{32}P-labelled fragment as described above.

C. Analysis of G418-resistant ES colonies

In the first experiment, PCR analysis of the pooled colonies detected one positive PCR signal of the expected size (about 1.4 kb) out of 34 pools representing 136 G418-resistant colonies. The four individual colonies that had contributed to this positive pool were analyzed individually by PCR, and a positive clone, ES33D5, was identified. Similar analysis of 540 G418-resistant colonies obtained in the second experiment yielded 4 additional positive clones (ES41-1, ES61-1, ES65-1, ES110-1).

In order to verify the targeted disruption of one copy of the J genes, (the gene is autosomal and thus present in two copies), the PCR positive clones were expanded and genomic DNA was prepared, digested with HindIII or with SacI and analyzed by Southern analysis as described using the EcoRI-PstI probe.

The replacement of the J genes by insertion of the neomycin gene by an homologous recombination event results in a HindIII fragment, detectable with the EcoRI-PstI

probe, which is about 1.9 kb longer than the equivalent fragment in the native locus, due to the loss of two HindIII sites located in the deleted J gene region (see Figure 2C). Southern analysis of each of the 5 positive clones by HindIII digestion gave a pattern which indicated that one of the two copies of the heavy chain J genes had been disrupted. Three labelled fragments were detected: one fragment (about 760 bp), identical in size to that present in untreated cells at the same intensity, one fragment (about 2.3 kb) identical in size to that present in untreated cells, but of decreased intensity in the PCR positive clone, and an additional fragment about 4.2 kb, the size predicted for an homologous recombination event, present only in the PCR-positive clones. Similarly, the replacement of the J genes by the neomycin gene by an homologous recombination event results in a loss of one SacI site and the appearance of a fragment, detectable with the EcoRI-PstI probe, which is about 570 bp smaller than the equivalent fragment in the native locus (see Figure 2C). Southern analysis of the clones by SacI digestion gave the expected pattern of one native and one targeted allele: about 4.0 kb fragment, identical in size to that detected in untreated cells, but of decreased intensity in the 5 positive clones, and an additional fragment of about 3.4 kb, the size predicted for a targeted homologous recombination event, present only in the identified clones. Rehybridization of the Southern blots with a probe for the neomycin gene showed that only the 4.2 kb and 3.4 kb fragments, resulting from the HindIII and the SacI digestion, respectively, hybridized to the probe as predicted by the targeting event.

D. Generation of chimeric mice with J_H deletions

Three and a half day old C57BL/6J (Jackson Laboratories, Bar Harbor, ME) blastocysts were obtained from 4-5 week old superovulated females as described by Koller, et al. 1989 (supra). ES cells were trypsinized,

washed once with fresh DMEM media, and diluted to about 1 X 10^6/ml in DMEM medium containing 10% fetal bovine serum and 20 mM HEPES, pH 7.5. 10 to 15 cells were injected into the blastocoel of each blastocyst. ES-cell containing blastocysts were then surgically transferred to one uterine horn of C57BL/6J X DBA/2 or C57BL/6J X CBA F1 pseudopregnant females.

The contribution of ES cells to the offspring was judged visually by examination of the coat color of the pups. C57BL/6J mice are solid black in color. The ES cell parent line E14TG2a was isolated from 129/Ola embryos, which carry three coat color genes, the dominant A^W allele at the agouti locus, the recessive pink-eyes-dilute allele at the p locus, and the recessive C^{ch} at the c locus. Chimeric offspring in which the ES cells participated in the formation of the animal have coats containing agouti and cream hairs.

Germline transmission ability of the chimeric mice was evaluated by mating with a C57BL/6J mouse and scoring for F1 offspring with agouti color. 50% of these agouti mice would be expected to inherit the mutated heavy chain allele, which can be identified by Southern blot analysis of DNA isolated from tails.

The J_H-targeted ES cell line ES65-1, carrying one targeted heavy chain allele, was injected into C57BL/6J mouse blastocysts. About 45% of the surviving pups were chimeras. Two chimeric females, 238-2 and 244-3, upon mating with C57BL/6J males, yielded germline transmission at a frequency of 100% and 15%, as determined by the percent of agouti offspring. Southern blot analysis of DNA from heterozygous offspring indicated the presence of the targeted heavy chain in addition to one native allele in 2 out of 5 agouti progeny tested.

Mice homozygous for the mutation were obtained by intercrossing male and female mice which were identified as J_H-deleted (δJ_H) heterozygotes. Offspring of these

matings were analyzed for the presence of the two targeted heavy chain alleles by Southern blot analysis.

E. Analysis of B cells from chimeric mice

If deletion of the J_H region is sufficient to inactivate the heavy chain locus, then it should result in complete block of development of IgM-expressing B cells and of antibody production. Mice which are heterozygous at the J_H locus carry one intact and functional heavy chain allele, derived from the C57BL/6J parent, and one J_H-deleted heavy chain allele which is derived from the ES cells (129/Ola strain). The 129 and B6 strains differ in Ig heavy chain allotypes. The ES-derived B cells (IgM^a allotype) can be distinguished from B6-derived B cells (IgM^b allotype) with allotype-specific monoclonal antibodies, using flow cytometry analysis of antibody expressing B.

The specificity of these antibodies is shown in Figure 3 (A-C). Peripheral blood lymphocytes were stained with antibodies to the B cell specific marker, B220, and with antibodies to the IgM allotype. B cells from C57BL/6J mice stained with antibodies directed against the IgM^b allotype but not the IgM^a allotype (Figure 3B). B cells derived from 129/Ola mice stained with antibody against the IgM^a allotype, but not the IgM^b allotype (Figure 3A). In heterozygous (a/b F1) mice carrying one intact ES-derived heavy chain allele and one intact C57BL/6J-derived heavy chain allele, both allotypes were present in equal amounts (Figure 3C).

When B cells from mice which were heterozygous for the J_H deletion were analyzed, where the J_H deleted heavy chain allele was from the 129/Ola parent, there were no cells positive for the IgM^a allotype. All B cells were IgM^b positive, from the intact C57BL/6J heavy chain allele (Figure 3D). These results indicated that the J_H-deleted heavy chain locus is inactivated and cannot encode a functional IgM antibody.

Mice which were homozygous for the J_H deletion were also analyzed for the ability to produce functional antibodies. Peripheral blood lymphocytes from homozygous mutant mice were analyzed by flow cytometry, using antibodies to the B cell specific marker B220, and with the allotype specific markers (see Figure 4). In contrast to the control mice (Figure 4D-F), no $B220^+$ cells, or IgM producing cells could be detected in the mutant mice (Figure 4A-C). In addition, the mutant mice had no detectable IgM in the serum. These results indicate that the deletion of the J_H region from both heavy chain alleles leads to complete inhibition of B cell development to mature B cells and production of antibody.

F. Generation of homozygous mutant ES cells

The effect of J_H deletion on B cells can also be analyzed by generating ES cells with both heavy chain alleles targeted, which are then used to produce chimeric mice which contain a population of lymphoid cells homozygous for the mutation.

Homozygous δJ_H mutant ES cells were generated by subjecting one of the heterozygous mutant ES clones, ES110-1, to elevated levels of G418 (1.4 mg/ml) thus selecting for homogenotization of the targeted allele. Seven of the surviving colonies were screened by Southern blot analysis using SacI digestion for the loss of the wild-type heavy chain allele and acquisition of a second targeted allele. One of these clones, ESDK207 was shown to have lost the native heavy chain allele, as evidenced by the inability of probes to detect the wild type 4.0 kb fragment and by the increased intensity of the 3.4 kb targeted fragment. Karyotypic analysis of ESDK207 indicated that, like the parent line ES110-1, about 80% of the cells had 40 chromosomes, suggesting that two targeted alleles were present. The homozygous mutant ES cells were microinjected into C57BL/6J blastocysts and chimeric mice were generated.

G. Analysis of B cells from homozygous chimeras

B cells from chimeric mice were analyzed to determine the effect of J_H deletion on B cell development and antibody production. Lymphocytes from the ES cell line (129/Ola) can be distinguished from blastocyst-derived (C57BL/6J) lymphocytes by a monoclonal antibody to the Ly-9.1 marker, which is found on lymphocytes of 129 origin, but not those of B6 origin. In addition, the two strains differ in their IgM allotype, as previously described.

The chimeras analyzed had been derived from wild-type E14TG2a ES cells (WT), or from ES cells that were heterozygous (ES110-1, ES65-1) or homozygous (ESDK207) at the targeted J_H region. Peripheral blood mononuclear cells were stained with antibodies to the B cell specific marker B220, and with antibodies to either Ly-9.1 or IgM allotypes, and then analyzed by two-color flow cytometry. To evaluate chimerism in the T cell lineage, the cells were stained with antibody for the T cell marker Thy 1.2, and with anti-Ly-9.1 antibody. Staining of cells from the parental mouse strains provided controls for the specificity and sensitivity of the assay.

Mice with similar degrees of chimerism, as judged by coat color, were compared. ES-derived B and T cells were detected in the peripheral blood of chimeric mice generated from the wild-type E14TG2a ES cells, confirming the ability of this cell line to give rise to lymphoid cells in vivo. Analysis of chimeras generated from single J_H-targeted ES65-1 and ES110-1 cells demonstrated the presence of $B220^+/IgM^{a+}/Ly\text{-}9.1^+$ B cells containing a single, intact, ES cell-derived Ig heavy chain locus.

In contrast to the WT and single deletion chimeras, mice generated from the homozygous mutant ESDK207 cell line lacked $Ly\text{-}9.1^+/B220^+$ or $IgM^{a+}/B220+1$ B cells in peripheral blood. The observed lack of ESDK207-derived B cells was not due to a lack in lymphopoiesis, since ES-derived $Ly\text{-}9.1^+/B220^-$ cells represented 12% of the total

pool of peripheral blood mononuclear cells. Of these, approximately half were Thy-1.2$^+$ T cells. Thus, deletion of the J_H region from both alleles blocks development of mature IgM^a producing B cells. Similar observations were made for chimeric spleen cells.

Chimeras were also tested for the presence of serum IgM derived from the ES cells. IgM^a levels were high in chimeras from wild-type ES cells and cells with a single targeted mutation, but were undetectable in mice derived from the ESDK207 cell line.

Further analysis showed that the bone marrow of ESDK207 mice contained normal IgM^{b+} B cells derived from the blastocyst host, but lacked ES-derived IgM^{a+} B cells. However, DK207-derived bone marrow did contain a population of cells which were B220^{dull}/Ly-9.1$^+$ derived from the ES cells. The bone marrow is therefore likely to contain a subpopulation of ES cell-derived B cell precursors, whose maturation is blocked by the homozygous deletion of the J_H region.

The bone marrow cells were also analyzed with three-color flow cytometry, using antibodies to Ly-9.1, B220 and either CD43 or Thy-1.2. The results show the majority of ES-derived cells were CD43 positive, which is consistent with an early block in maturation. Many of the cells were also positive for Thy-1.2, as would be expected of very early B cell precursors. These data show that deleting the J_H region results in the inability of the heavy chain locus to rearrange and produce functional IgM. Lack of IgH rearrangement results in a block of B cell maturation, restricting B cell progenitors to an early stage of development.

EXAMPLE III

Deletion of the Mouse Ig kappa light chain constant (C_k) region

A. Construction of the replacement targeting vector

The kappa region was inactivated with a replacement type vector, which was designed to delete the constant region of the kappa locus, and replace it with the G418 drug resistance marker through homologous recombination. Homologous recombination was driven by regions of homology which flank the constant region (see Figure 5).

A genomic library from 129/Ola mouse fetal liver DNA (Stratagene) cloned into lambda phage was screened for the presence of the mouse C_k gene with a 1.6 kb HpaI/BamHI fragment (Steinmetz and Zachau (1980) Nucleic Acids Research 8:1693-1706) that spans the mouse kappa constant region. A lambda phage clone which hybridized to this probe was identified, then purified and used as a source of C_k DNA. Analysis of the phage DNA showed that the kappa constant region probe hybridized to a 5.6 kb SphI/BamHI fragment. This fragment contained the kappa J region genes, an intronic enhancer element and the kappa constant region. It was then isolated and subcloned into the SphI and BamHI sites of the plasmid pUC218 to give the plasmid pUC218/5.6kappa.

In order to construct the deletion vector, fragments containing the 5′ region of the kappa constant region, a thymidine kinase gene for negative selection, a neomycin resistance gene and a 3′ region of homology to the kappa constant region were ligated together (see Figure 6).

A 4.0 kb SphI/Bsu361 fragment from the plasmid pUC218/5.6kappa was subcloned into the SphI and Bsu361 sites of the vector pSK.A to give the plasmid pSK.A/5′K. The vector pSK.A is a modification of pBluescript SK- which has a synthetic polylinker:

5′ GCATATGCCTGAGGTAAGCATGCGGTACCGAATTCTATAAGCTTGCGGCCGCAGCT
CATGCGTATACGGACTCCATTCGTACGCCATGGCTTAAGATATTCGAACGCCGGCG 3′

inserted between the pBluescript KpnI and SacI sites.

A 2.7 kb EcoRI/HindIII fragment containing the herpes thymidine kinase (TK) gene driven by the mouse phosphoglycerate kinase gene (PGK) promoter from the plasmid pKJtk (Tybulewicz, et al. (1991) Cell 65:1153-1163) was inserted into the EcoRI and NotI sites of pSK.A/5'K by using a HindIII/NotI adapter with the sequence:

5' AGCTGGAACCCCTTGCCCTTGGGGAACGCCGG 3'.

In the resulting plasmid, pSK.A/5'K/TK, the 5' end of the TK gene and the kappa constant region gene are adjacent to each other, in opposite transcriptional orientations.

A 1.1 kb XhoI/BamHI fragment from pMC1Neo, which contains the mammalian drug selectable marker for resistance to neomycin, was cloned into the XhoI and BamHI sites of the plasmid pSK.B to give the plasmid pSK.B/Neo. The vector pSK.B is a modification of pBluescript SK- which has a synthetic polylinker:

5' GAGCTCGGATCCTATCTCGAGGAATTCTATAAGCTTCATATGTAGCT
CATGCTCGAGCCTAGGATAGAGCTCCTTAAGATATTCGAAGTATACA 3'

inserted between the pBluescript KpnI and SacI sites.

A 1.1 kb BglII/BamHI fragment from pUC218/5.6kappa, which contains homology to the 3' end of the kappa region, was cloned into BamHI digested, alkaline phosphatase treated pSK.C vector. The vector pSK.C is a modification of pBluescript SK- which has a synthetic polylinker:

5' AAGCTTATAGAATTCGGTACCTGGATCCTGAGCTCATAGCGGCCGCAGCT
CATGTTCGAATATCTTAAGCCATGGACCTAGGACTCGAGTATCGCCGGCG 3'

inserted between the pBluescript KpnI and SacI sites. The resulting plasmid, pSK.C/3'K is oriented such that transcription proceeds from the SacI site in the plasmid polylinker in the direction of the KpnI site.

The final targeting plasmid was constructed with a three part ligation, using (A) 6.1 kb NotI/NdeI fragment from pSK.A/5'K/TK, (B) 1.2 kb NdeI/SacI fragment from pSK.B/Neo and (C) 4.0 kb SacI/NotI fragment from pSK.C/3'K ligated to make the plasmid pK.TK/neo.

B. Electroporation of kappa deletion vector into ES cells

Purified plasmid DNA from pK.TK/Neo was cut with PvuI, extracted with phenol/chloroform and ethanol precipitated. The DNA was resuspended after precipitation at a concentration of 1 mg/ml in 10 mM Tris-HCl, 1 mM EDTA.

The embryonic stem cell line E14-1, a subclone of E14 (Hooper, et al. (1987) Nature 326:292-295) was cultured in DMEM 4.5 g/l glucose (J.R.H. Biosciences) supplemented with 15% heat inactivated fetal calf serum, recombinant murine leukemia inhibitory factor (ESGRO from Gibco BRL, 1000 U/ml), 0.1 mM β-mercaptoethanol, 2 mM glutamine and 100 U/ml penicillin at 37° C in 5% CO_2.

The cells were cultured on mitomycin-treated primary embryonic fibroblast feeder layers essentially as described (Koller and Smithies (1989) supra). The embryonic fibroblasts were prepared from day 14 embryos carrying the homozygous targeted mutation of β2-microglobulin (Koller and Smithies (1990) Science 248:1227-1230). These feeder cells are capable of growth in media containing G418.

At 80% confluency, the ES cells were prepared for electroporation by trypsinization, concentration by brief centrifugation and resuspension in HEPES-buffered saline at 2 x 10^7 cells/ml. The cells are equilibrated at room temperature, and linearized targeting vector DNA (20 μg) added. The mixture was electroporated at 960 μF and 250 V with a BioRad Gene Pulser. The cells were left to stand at room temperature for 10 minutes before plating onto 4 x 10 cm dishes of mitomycin-treated fibroblast feeders (3 x 10^6 feeder cells/plate). After incubation at 37° C for 48 hours, the cells were fed media containing 150 μg/ml G418 to select for neomycin resistance. After a further 48 hours the cells were fed media containing 150 μg/ml G418 and 2 μM gancyclovir (Syntex) to select for loss of the thymidine kinase gene.

C. Analysis of targeted ES cells

After ten days of drug selection with both G418 and gancyclovir, the individual surviving colonies were picked and dissociated with a drop of trypsin in a 96 well plate, then incubated at 37° for 2 minutes. The cells from each colony were transferred into a well of a 24-well plate containing mitomycin C-treated feeder cells and selective media with G418, but not gancyclovir. After an additional 5-8 days, 20% of the cells in each well were frozen, and the remainder used to prepare genomic DNA. The cells were lysed with 0.4 ml of 10 mM Tris-HCl (pH 7.5), 100 mM NaCl, 10 mM EDTA, 1% SDS and proteinase K (1 mg/ml) by overnight incubation at 50° C. The DNA was purified by phenol extraction and ethanol precipitation, then washed with 70% ethanol and resuspended in 20 μl of 10 mM Tris-HCl, 1 mM EDTA.

Southern analysis was carried out using BglII digested genomic DNA from each sample. An about 1.2 kb BamHI/BglII fragment which contains the region contiguous with the 3′ homology fragment in the targeting vector was used as a probe. The native ES cell locus gave an about 2.3 kb fragment, while the targeted ES cell locus gave an about 5.7 kb fragment. The increase in size is due to the loss of a BglII site during the construction of the deletion vector.

A Southern analysis of 166 clones showed two cell lines which had the intended mutation. These clones were further analyzed by reprobing the filters with an about 1.1 kb fragment which spans the neo gene. As expected, the probe only hybridized to the targeted allele.

Further analysis of the genomic DNA from the two positive clones, 1L2-850 and 1L2-972, after being thawed and expanded, reconfirmed the initial observations. A third probe, an about 1.7 kb HindIII/BglII fragment spanning the kappa J region locus, was used to check for the correct integration pattern from the 5′ end of the targeting vector. Using this probe with EcoRI digested

genomic DNA, an about 15 kb fragment is detected in the native allele, and an about 5 kb fragment from the targeted locus. The additional EcoRI site is introduced by the neo gene during homologous recombination targeting (see Figure 7).

D. Generation of germline chimeras

The unmodified E14-1 cells have been found to contribute to the germline at a high frequency after injection into C57BL/6J blastocysts. To generate germline chimeras containing the targeted kappa region, the targeted cell lines 1L2-850 and 1L2-972 were grown on primary feeder cells, then trypsinized and resuspended in injection medium, which consists of DMEM supplemented with 15% fetal calf serum, 20 mM HEPES (pH 7.3), antibiotics and β-mercaptoethanol. The ES cells were injected into each blastocyst, and the injected blastocysts then transferred to one uterine horn of a pseudopregnant female mouse. Chimeric pups were identified by chimeric coat color. Chimeric males were bred to C57BL/6J females, and germline transmission of the 129/Ola derived ES cells was detected by agouti coat color of the offspring.

One chimeric male from cell line 1L2-972 (about 40% ES cell derived as judged by its coat color), upon mating with C57B1/6J females yielded germline transmission at a frequency of 25% as determined by the percent of agouti offspring. Chimeric males, about 40%, 70% and 90% chimeric, from cell line 1L2-850 yielded germline transmission at a frequencies of 90%, 63% and 33%, respectively. Among the agouti offspring generated from the 70% chimeric male from 1L2-850, eight F1 animals out of 12 tested were found to be heterozygous at the kappa locus for the targeted C_K mutation by Southern analysis (a Bgl II digest using the 1.2 kb Bam HI/Bgl II fragment described above as a probe) using genomic DNA derived from tail samples. Further breeding of a male and female from

this group of 8 F1 animals, both heterozygous for the C_K mutation, yielded one male offspring found to be homozygous for this mutation as confirmed by Southern analysis.

E. Analysis of B cells obtained from mice targeted at the kappa locus

If the kappa (κ) light chain locus is inactivated because of deletion of the light chain constant region (Cκ), the joining region(Jκ), or both Cκ and Jκ, then a complete block in the development of κ-expressing B cells should result. Mouse embryonic stem cells containing a single copy of the complete Cκ deletion (ΔCκ) were introduced into mouse blastocysts as described above to produce chimeric mice. These chimeric mice were then bred with wild-type C57BL/6 (B6) mice, and the F1 progeny were assayed for the presence of the ΔCκ mutation by Southern blotting of tail DNA. F1 mice that carried the ΔCκ mutation were bred and F2 offspring were assayed similarly for ΔCκ. One of 5 F2 offspring was shown to carry a homozygous Cκ deletion, and another was heterozygous, bearing both ΔCκ and a wild-type Cκ allele. The 3 other offspring were wild-type. The presence or absence of κ-positive B cells was assayed by flow cytometric analysis of peripheral blood B cells stained with fluorescent antibodies that react with a pan-B cell marker (B220) or with the κ light chain. For the homozygous ΔCκ F2 mouse no κ-positive B cells were detected, and in the heterozygote, there was a reduction in the frequency of κ positive B cells, consistent with the presence of a wild-type allele and a non-functional ΔCκ allele. These results demonstrate that deletion of Cκ from the chromosome prevents κ expression by mouse B cells.

EXAMPLE IV

Inactivation of the mouse immunoglobulin kappa light chain J and constant region

A. Design of the targeting experiment

The targeting vector was designed as a replacement type vector initially to delete the constant region as well as the J region of the kappa locus and replace it with three elements through homologous recombination using regions of homology flanking the constant region (Figure 8). A diphtheria toxin gene (A chain) flanking either or both regions of homology was included in some cases as a negative selectable marker. The three elements consisted of the G418 resistance drug marker, an additional DNA homology (ADH) sequence of mouse DNA homologous to a region of the kappa locus located upstream of the J region, and a thymidine kinase gene. As a result of the inclusion of the ADH sequence in the vector, this initial targeting placed a second copy of the ADH in the locus. This duplication was then used to effect a defined deletion of the sequences between the segments by applying selective pressure. In this case the cell deletes the thymidine kinase gene that lies between the two segments in order to survive gancyclovir selection.

B. Construction of the targeting vector

The regions of homology were derived from a 129 mouse fetal liver genomic library (Stratagene) which was screened using two probes, as described above in Example III. This subclone contained the J region, an intronic enhancer element and the constant region of the kappa light chain locus. The second probe was a 0.8 kb EcoRI fragment (Van Ness et al. (1981), Cell 27:593-602) that lies 2.8 kb upstream of the J region. Phage DNA from a lambda clone positive for this probe showed that the probe hybridized to a 5.5 kb SacI fragment which was subcloned into the SacI site of pBluescript SK (Stratagene) to give the plasmid pSK.5'kappa (Figure 8).

The inactivation vectors which contained a 5′ region of homology, a thymidine kinase gene, a ADH, a neomycin resistance gene and a 3′ region of homology (Figure 9) flanked in some instances by diphtheria toxin genes were constructed from three plasmids (Figure 8) containing: (a) the 5′ fragment of homology with or without the diphtheria toxin gene (DT) driven by the mouse phosphoglycerate kinase gene (PGK) promoter as a negative selectable marker, (b) the herpes thymidine kinase gene (tk) driven by the mouse phosphoglycerate kinase gene (PGK) promoter as a negative selectable marker along with the DSH and the G418 selectable neomycin (neo) gene from pMC1Neo (Thomas and Capecchi (1987), Cell 51:503-12), and (c) the 3′ fragment of homology with or without the PGK driven DT gene. These three plasmids (Figure 8) were constructed from pSK.A, pSK.B, and pSK.C, respectively, all derived from the plasmid pBluescript SK⁻ by modification of the polylinker.

The polylinker of the plasmid pBluescript SK⁻ was modified by cloning between the KpnI and SacI sites a synthetic polylinker defined by the oligonucleotides 5′-GCATATGCCTGAGGGTAAGCATGCGGTACCGAATTCTA TAAGCTTGCGGCCGCAGCT-3′ AND 5′-GCGGCCGCAAGCTTATAGAATTC GGTACCGCATGCTTACCTCAGGCATATGCGTAC-3′ to create the plasmid pSK.A, 5′- GAGCTCGGATCCTATCTCGAGGAATTCTATAAGCTTCATATGT AGCT-3′ and 5′-ACATATGAAGCTTATAGAATTCCTCGAGATAGGATCCHA GCTCGTAC-3′ to create plasmid pSK.8, 5′-AAGCTTATAGAATTCGGTACC TGGATCCTGAGCTCATAGCGGCCGCAGCT-3′ to create plasmid psK.B and 5′-GCGGCCGCTATGAGCTCAGGATCCAGGTACCGAATTCTATAAGCTTG TAC-3′ to create the plasmid pSK.C.

A diphtheria toxin gene cassette was created in which the gene was flanked by the PGK promoter and the bovine growth hormone polyadenylation signal (Woychik et al. (1984), Proc. Natl. Acad. Sci. U.S.A, 81:3944-3948; Pfarr et al. (1986), DNA 5:115-122). A 2.3 kb XbaI/EcoRI fragment from pTH-1 (Maxwell et al. (1986), Cancer Res.

46:4660-4664) containing the diphtheria toxin A chain driven by the human metallothionein (hMTII) promoter was cloned into pBluescript SK cut with XbaI and EcoRI to give the plasmid pSK.DT. The hMTII promoter of pSK.DT was replaced with the PGK promoter from pKJ1 (Tybulewicz et al. (1991), Cell 65:1153-1163). A 0.5 kb XbaI/PstI fragment from pKJ1 was joined to a 3.1 kb XbaI/NcoI fragment from pSK.DT using a PstI/NcoI adapter formed from the oligonucleotides 5'-GGGAAGCCGCCGC-3' and 5'-CATGGC GGCGGCTTCCCTGCA-3' to give the plasmid pSK.pgkDT. A 248 bp fragment containing the bovine growth hormone polyadenylation signal, obtained by PCR amplification of bovine genomic DNA using the oligonucleotide primers 5'-CAGGATCCAGCTGTGCCTTCTAGTTG-3' and 5'-CTGAGCTCTAGACCCATA GAGCCCACCGCA-3', was cloned into pCR1000 (Invitron Corp., San Diego, CA). The polyadenylation sequence was then cloned behind the DT gene as a HindIII/PvuII fragment into pSK.pgkDT cut with HindIII and HpaI to give the plasmid pSK.pgkDTbovGH. The DT gene cassette from pSK.pgkDTbovGH was moved as a 2.1 kb EcoRI/HindIII fragment into pSK.A cut with EcoRI and NotI using a HindIII/NotI adapter formed from the oligonucleotides 5'-AGCTGGAACCCCTTGC-3' and 5'-GGCCGCAAGGGGTTCC-3' to give the plasmid pSK.A/DT. Between the SphI and Bsu36I sites of both pSK.A and pSK.A/DT the 5' region of homology for the kappa locus was cloned. For this purpose a 4.0 kb SphI/Bsu36I fragment resulting from a partial Bsu36I digest followed by a complete SphI digest of plasmid subclone pUC218/5.6kappa was ligated to pSK.A or pSK.A/DT to give the plasmids pSK.A/5'K and pSK.A/DT/5'K, respectively. In the plasmid, pSK.A/DT/5'K, the 5'-end of the DT gene and kappa fragment were adjacent to each other running in the opposite transcriptional orientations.

The PGKtk gene from the plasmid pKJtk (Tybulewicz et al. (1991), Cell 65:1153-1163) was cloned as a 2.7 kb EcoRI/HindIII between the unique EcoRI and HindIII sites of pSK.B to give pSK.B/TK. A 0.8 kb EcoRI fragment used

for the ADH was cloned from pSK.5'kappa and was ligated into the EcoRI site of pSK.B/TK to give pSK.B/(TK/0.8K) such that the 5'-end of the tk gene and kappa fragment were adjacent to each other running in opposite transcriptional orientations. The 1.1 kb neo gene from pMC1Neo was cloned as an XhoI/BamHI fragment between the same sites of pSK.B/(TK/0.8K) to give pSK.B/(TK/0.8K/Neo). The plasmid pSK.C/3'K containing the 3' fragment of homology was constructed by ligating pSK.C digested with BamHI and treated with alkaline phosphatase to the 1.1 kb Bg1II/BamHI fragment isolated from pUC218/5.6kappa. In pSK.C/3'K, the kappa fragment was oriented such that transcription proceeded from the SacI in the plasmid polylinker in the direction of the KpnI site. The 2.1 kb DT cassette from pSK.pgkDTbovGH was cloned as an EcoRI/HindIII fragment into the same sites of pSK.C to give pSK.C/3'K/DT.

Three-part ligations were carried out to construct the final targeting plasmids (Figure 9). The 4.0 kb NotI/NdeI fragment from pSK.A/5'K, the 4.8 kb NdeI/SacI fragment from pSK.B/(TK/0.8K/Neo) (obtained by a SacI partial followed by and NdeI digestion of the plasmid), and the 4.0 kb SacI/NotI fragment from pSK.C/3'K were isolated and ligated together to create pK.(TK/0.8K/Neo). The 6.1 kb NotI/NdeI fragment from pSk.A/DT/5'K, the 4.8 kb NdeI/SacI fragment from pSK.B/(TK/0.8K/Neo), and 4.0 kb SacI/NotI fragment from pSK.C/3'K were isolated and ligated together to create pK.DT/(TK/0.8K/Neo). The 6.1 kb NotI/NdeI fragment from pSK.A/DT/5'K, the 4.8 kb NdeI/SacI fragment from pSK.B/(TK/0.8K/Neo), and 6.1 kb SacI/NotI fragment from pSK.C/3'K/DT (obtained by a SacI partial followed by a NotI digestion of the plasmid) were isolated and ligate together to create pK.DT/(TK/0.8K/Neo)/DT. For electroporation, the purified plasmid DNAs were first cut with PvuI or ApaLI, then extracted with phenol/chloroform and precipitated by the addition of ethanol before centrifugation. The resultant

DNA pellets were resuspended at a concentration of 1 mg/ml in 10 mM Tris-HCl, 1 mM EDTA(TE).

C. Introduction of DNA into cells

The embryonic stem cell line E14-1 was cultured as described above in Example III. The cells were equilibrated at room temperature, and DNA (20 μg) linearized with PvuI (as described above) was added. The mixture was electroporated as described above in Example III.

D. Analysis of constant region-targeted ES cells

After 7-10 days under drug selection with G418, the individual surviving colonies were each picked and dissociated in a drop of trypsin as described above in Example III.

Southern analysis was carried out using BgIII digested genomic DNA from each sample. A 2.3 kb fragment was detected from the native ES cell locus, while a larger 4.9 kb fragment was detected from a targeted ES cell locus (Figure 11), using as a probe the 1.2 kb BamHI/BgIII fragment isolated from the original phage DNA contiguous with the fragment used for the 3′ homology in the targeting vector. The fragment increased in size because the BgIII site in the BgIII/BamHI fragment was lost in the targeting plasmid due to the joining of a BgIII site to a BamHI site in the ligation, and a new BgIII site located in the thymidine kinase gene is introduced into the targeted locus.

From a screen by the Southern analysis described above, of a total of 103 clones derived from experiments using three different targeting plasmids, 5 cell lines were identified which carried the intended mutation (Table 1).

Table 1

C_K Light Chain Targeting Result in E14-1

Construct	Number Screened by Southern	Number of Confirmed Targeted Clones	Clone Designation	Frequency of Targeting
pK.(TK/0.8K/Neo)	44	2	625,691	1/22
pK.DT(TK/0.8/Neo)	42	2	604,611	1/21
pK.DT(TK/0.8K/Neo)DT	17	1	653	1/17

Further analysis of genomic DNA produced from 4 of the positive clones (clones 625, 604, 611 and 653) after being thawed and expanded, re-confirmed the initial observations. Using a second probe, a 1.7 kb HindIII/BgIII fragment which spanned the J region of the kappa locus, the correct integration pattern was checked for homologous targeting at the 5' end of the targeting vector. Thus, using this probe with an EcoRI digest of the genomic DNA, a 15 kb fragment was detected from the unmodified allele. In contrast, a 7.8 kb fragment from the targeted allele was observed as a result of the introduction of a new EcoRI site in the thymidine kinase gene during the homologous integration (Figure 11).

E. In vitro excision of J region DNA from targeted clones

In order to effect the desired deletion from the homologously targeted kappa locus, cells from clone 653 were plated on feeder cells at a density of 0.5-1 x 10^6 cells/10 cm dish in the presence of both gancyclovir (2 μM) and G418 (150 μg/ml). After growth for 5 days in the presence of both drugs, clones were picked as described above into 24-well plates and grown under G418 selection alone. After an additional 5-8 days, 20% of the cells in each well were frozen and the remainder used to prepare genomic DNA as previously described.

F. Analysis of J/constant region deleted ES cells

Southern analysis was carried out using BamHI digested genomic DNA from each sample. Using as a probe the 0.8

kb EcoRI fragment used as the ADH in the targeting vectors, as 12.7 kb fragment was detected from the native ES cell locus, while a larger 15.8 kb fragment was detected from the constant region-targeted ES cell locus (Figure 11) using DNA from clone 653. The fragment increased in size because of the insertion of the tk gene, the ADH, and the neo gene into the 12.7 kb BamHI fragment. There was also a new BamHI site introduced at the 3′ end of the neo gene. Using DNA from the J/constant region deleted cells, a 5.5 kb fragment was detected from the modified locus in addition to the 12.7 kb fragment from the untargeted allele as predicted from analysis of the restriction map. From this screen by Southern analysis of 2 clones produced from 1.5 x 10^6 ES cells plated (clone 653), one cell line (clone 653B) was identified which carried the intended deletion of the J and constant regions.

Further analysis of genomic DNA produced from clone 653B after being thawed and expanded re-confirmed the initial observations. Using the 0.8 kb EcoRI fragment, the deletion was checked with two other restriction digests which should cut outside of the excised region on the 5′ and 3′ ends of the targeting vector. Thus using this probe with a BgIII digest of the genomic DNA from the unexcised clone 653, a 2.6 kb fragment was detected from both the unmodified and modified alleles, whereas an additional 4.9 kb fragment was observed from the targeted allele only (Figure 11). This 4.9 kb fragment was the same as that detected with the 1.2 kb BamHI/BgIII fragment used previously. Using DNA from clone 653B, a BgIII digest revealed a 5.8 kb fragment in addition to the 2.6 kb fragment from the unmodified allele. A SacI digest of clone 653 DNA probed with the 0.8 kb EcoRI fragment showed a 5.5 kb fragment from both the unmodified and modified alleles and a 3.1 kb fragment from the targeted allele only (Figure 11). The 5.5 kb fragment was also detected in DNA from clone 653B and an additional

2.0 kb fragment. The 5.8 kb BgIII fragment and the 2.0 kb ScaI fragment were consistent with an analysis of the predicted restriction map for a precise excision step in which 10.3 kb of DNA were deleted including the J region, the tk gene, and one copy of the ADH.

G. Generation of Germline Chimeras

The unmodified E14-1 cells contributed to the germline at a high frequency after injection into C57BL/6J blastocysts. The cells from the targeted ES cell line 691, in which only the kappa constant region has been deleted by homologous recombination without any negative selection, were microinjected and chimeric animals were produced as described above in Example III. Cells from the targeted ES cell line 653B in which both the kappa constant and J regions were deleted are also microinjected and chimeric animals are produced as described above. Chimeric pups are identified by chimeric coat color. Germline transmission of the modified ES cell is detected by the agouti coat color of the F1 offspring.

EXAMPLE V

Cloning of Human Heavy Chain Locus using Yeast Artificial Chromosomes

A. Production of Yeast Artificial Chromosome (YAC) containing human heavy chain

An SpeI fragment, spanning the human heavy chain VH6-D-J-Cμ-Cδ region (Berman et al. (1988), EMBO J. 7: 727-738; see Figure 15) is isolated from a human YAC library (Burke, et al., Science, 236: 806-812) using DNA probes described by Berman et al. (1988) EMBO J. 7:727-738. One clone is obtained which is estimated to be about 100 kb. The isolated YAC clone is characterized by pulsed-field gel electrophoresis (Burke et al., supra; Brownstein et al., Science, 244: 1348-1351), using radiolabelled probes for the human heavy chain (Berman et al., supra).

B. Introduction of YAC clones into embryos or ES Cells

High molecular weight DNA is prepared in agarose plugs from yeast cells containing the YAC of interest (i.e., a YAC containing the aforementioned SpeI fragment from the IgH locus). The DNA is size-fractionated on a CHEF gel apparatus and the YAC band is cut out of the low melting point agarose gel. The gel fragment is equilibrated with polyamines and then melted and treated with agarase to digest the agarose. The polyamine-coated DNA is then injected into the male pronucleus of fertilized mouse embryos which are then surgically introduced into the uterus of a psueudopregnant female as described above. The transgenic nature of the newborns is analyzed by a slot-blot of DNA isolated from tails and the production of human heavy chain is analyzed by obtaining a small amount of serum and testing it for the presence of Ig chains with rabbit anti-human antibodies.

As an alternative to microinjection, YAC DNA is transferred into murine ES cells by ES cell: yeast protoplast fusion (Traver et al., (1989) Proc. Natl. Acad. Sci., USA, 86:5898-5902; Pachnis et al., (1990), ibid 87: 5109-5113). First, the neomycin-resistance gene from pMC1Neo or HPRT or other mammalian selectable marker and a yeast selectable marker are inserted into nonessential YAC vector sequences in a plasmid. This construct is used to transform a yeast strain containing the IgH YAC, and pMC1Neo (or other selectable marker) is integrated into vector sequences of the IgH YAC by homologous recombination. The modified YAC is then transferred into an ES cell by protoplast fusion (Traver et al. (1989); Pachnis et al., 1990), and resulting G418-resistant ES cells (or exhibiting another selectable phenotype) which contain the intact human IgH sequences are used to generate chimeric mice. Alternatively, a purified YAC is transfected, for example by lipofection or calcium phosphate-mediated DNA transfer, into ES cells.

EXAMPLE VI

Introduction of Human Ig Genes into Mice

A. Cloning of Human Ig Genes in Yeast

1. Identification and characterization of a human IgH YAC clone containing VH, D, JH, mu and delta sequences:

PCR primers for the human VH6 gene (V6A= 5′ GCA GAG CCT GCT GAA TTC TGG CTG 3′ and V6B= 5′ GTA ATA CAC AGC CGT GTC CTG G 3′) were used to screen DNA pools from the Washington University human YAC library (Washington University, St. Louis, MO). Positive pools were subsequently screened by colony hybridization and one positive microtiter plate well, A287-C10, was identified. Two different sized (205 kb and 215 kb) VH6-containing YACs were isolated from the microtiter well. In addition to VH6, the smaller of the two IgH YACs , A287-C10 (205 kb), hybridized to probes for the following sequences: delta, mu, JH, D, VH1, VH2, and VH4. The larger of the two IgH YACs, A287-C10 (215 kb), hybridized to the following probes: delta, JH, D, VH1, VH2, and VH4, but not to mu. The YACs contained sequences from at least 5 VH genes including two VH1 genes, one VH2, one VH4 and one VH6 gene. Analysis of restriction digests indicated that the 205 kb YAC contains a deletion (about 20 kb size) that removes some, but not all of the D gene cluster, with the remainder of the YAC appearing to be intact and in germline configuration. PCR and detailed restriction digest analysis of the 205 kb YAC demonstrated the presence of several different D gene family members. The 215 kb YAC appeared to contain the complete major D gene cluster but had a deletion (about 10 kb) that removed the mu gene. This deletion does not appear to affect the JH cluster or the enhancer located between JH and mu genes.

The putative progenitor of the above two related IgH YACs, a YAC of about 225-230 kb containing the entire genomic region between the VH2 gene and the delta gene (Shin et al., 1991, supra) (see Figure 15), had not been

identified in the A287-C10 microtiter well. Hence, an earlier aliquot of the A287-C10 microtiter plate well was examined in order to search for the progenitor YAC under the assumption that it was lost during passaging of the library. The A287-C10 microtiter well was streaked out (Washington University, St. Louis, MO), and 2 of 10 clones analyzed contained a 230 kb IgH YAC with another apparently unrelated YAC. Clone 1 contained in addition the IgH YAC, an approximately 220 kb YAC and clone 3 in addition contained an approximately 400 kb YAC. The IgH YAC contained mu, the complete D profile (based on a BamHI digest, see below) and JH. The IgH YAC from clone 1 was physically separated from the unrelated YAC by meiotic segregation in a cross between A287-C10/AB1380 and YPH857 (genotype = MATα ade2 lys2 ura3 trp1 HIS5 CAN1 his3 leu2 cyh2, to yield A287-C10 (230 kb)/MP 313 (host genotype = MATα ade2 leu2 lys2 his3 ura3 trp1 can1 cyh2).

2. Targeting of the A287-C10 kb YAC with a mammalian selectable marker, HPRT:

A YAC right arm targeting vector called pLUTO (15.6 kb) was generated by subcloning a human HPRT minigene contained on a 6.1 kb BamHI fragment (Reid et al., Proc. Natl. Acad. Sci. USA 87:4299-4303 (1990)) into the BamHI site in the polylinker of pLUS (Hermanson et al., Nucleic Acids Research 19:4943-4938 (1991)). A culture of A287-C10/AB1380 containing both the 230 kb IgH YAC and an unrelated YAC was transformed with linearized pLUTO and Lys+ transformants were selected. The Lys+ clones were screened by colony hybridization for the presence of mu. One clone was identified which contained a single YAC of approximately 245 kb which hybridized to probes for mu, HPRT and LYS2.

Southern analysis of the 230 kb A287-C10 YAC targeted with pLUTO was carried out using a variety of probes to demonstrate the intact, unrearranged nature of the cloned, human IgH sequences. In most cases, the results of BamHI,

HindIII and EcoRI digests were compared to restriction data for WI38 (a human embryonic fetal lung-derived cell line), the 205 kb and 215 kb deletion-derivatives of A287-C10 and to published values. The diversity (D) gene profile determined by hybridization with a D region probe (0.45 NcoI/PstI fragment; Berman et al., 1988) demonstrated the expected four D gene segments (D1-D4 (Siebenlist et al., 1981; Nature 294:631-635). For example, with BamHI, four restriction fragments, 3.8 kb, 4.5 kb, 6.9 kb and 7.8 kb, were observed in A287-C10 and WI38. WI38 had one additional larger band, presumed to originate from the chromosome 16 D5 region (Matsuda et al., 1988, EMBO 7:1047-1051). PCR and Southern analysis with D family-specific primers and probes demonstrated in the 215 kb deletion-derivative YAC (which appeared to have an intact D region with the same restriction pattern as the 230 kb YAC) the presence of 2 to 4 members of each of the following D gene families: DM, DN, DK, DA, DXP and DLR. The J-mu intronic enhancer, which was sequenced from cloned PCR products from the A287-C10 230 kb YAC (primers EnA = 5′ TTC CGG CCC CGA TGC GGG ACT GC 3′ and EnB1 = 5′ CCT CTC CCT AAG ACT 3′) and determined to be intact, also generated single restriction fragments of approximately the predicted sizes with BamHI, EcoRI and HindIII when probed with the 480 bp PCR product. The JH region was evaluated with an approximately 6 kb BamHI/HindIII fragment probe spanning DHQ52 and the entire JH region (Ravetch et al., 1981, Cell 27:583-591). A287-C10 generated restriction fragments of approximately the expected sizes. Furthermore, the same-sized restriction fragments were detected with the enhancer and the JH probes (Ravetch et al., supra; Shin et al., 1991, supra). The approximately 18 kb BamHI JH fragment detected in A287-C10 and WI38 also hybridized to a 0.9 kb mu probe sequence (Ravetch et al., supra). Hybridization with the 0.9 kb EcoRI fragment mu probe (Ravetch et al., supra) showed restriction fragments of approximately the expected

sizes (Ravetch et al., supra; Shin et al., supra): > 12 kb BamHI (approximately 17 kb expected); 0.9 kb EcoRI (0.9 kb expected) and approximately 12 kb HindIII (approximately 11 kb expected). WI38 gave the same-sized BamHI fragment as A287-C10. The JH and DHQ52 regions were sequenced from both of the deletion derivative YACs and both were in germline configuration. Delta was analyzed with an exon 1 PCR product (containing the approximately 160 bp region between primers D1B= 5′ CAA AGG ATA ACA GCC CTG 3′ and D1D = 5′ AGC TGG CTG CTT GTC ATG 3′); restriction fragments for A287-C10 were close to those expected from the literature (Shin et al., supra) and to those determined for WI38. The 3′ cloning site of the YAC may be the first EcoRI site 3′ of delta (Shin et al., supra) or another EcoRI site further 3′. VH gene probes for VH1, VH4 and VH6 (Berman et al., supra), and for VH2 (Takahashi et al., 1984, Proc. Nat. Acad. Sci. USA 81:5194-5198) were used to evaluate the variable gene content of the YAC. A287-C10 contains two VH1 genes that approximate the predicted sizes (Shin et al., supra; Matsuda et al., 1993, supra); restriction analysis with the three enzymes gave close to the expected fragment sies; e.g. with EcoRI observed bands are 3.4 and 7.8 kb (expected are 3.4 and 7.2 kb). The predicted size EcoRI fragments for VH4 (5.3 kb observed, 5.1 kb expected) and for VH6 (0.8 kb observed, 0.9 kb expected) (Shin et al., supra; Matsuda et al., supra) were present in A287-C10. The expected size EcoRI fragment was seen for VH2 (5.5 kb observed, 5.4 kb expected), but the BamHI and HindIII fragments were different from those predicted. Coincident hybridization of the BamHI and HindIII fragments with a pBR322 probe suggested that the EcoRI site which is at the 5′ end of the VH2 gene (Shin et al., supra) is the 5′ cloning site, thus eliminating the natural 5′ HindIII site and BamHI sites. The overall size of the YAC insert (estimated to be approximately 220 kb) fits well with the predicted size for an intact, unrearranged segment

starting at the 5′ end of the 3′-most VH2 gene and extending to an EcoRI site 3′ of the delta locus (Shin et al., supra).

3. Identification and characterization of IgK YACs containing CK and VK sequences:

Two YACs were identified in a screen of pulsed-field gel (PFG) pools from the Washington University (St. Louis, MO) human YAC library with a probe from the human kappa constant region (CK) gene (2.5 kb EcoRI fragment ATCC No. 59173, Parklawn Dr., Rockville, MD). The YACs, designated A80-C7 (170 kb) and A276-F2 (320 kb), contain the kappa deleting element kde, CK, JK and the C-J intronic enhancer and extend 3′ beyond kde. Extending 5′ from JK, the YACs also contain the B1, B2 and B3 VK genes determined by hybridization and/or PCR, and possibly other VK sequences. The A80-C7/AB1380 strain housed, in addition to the IgK YAC, an unrelated YAC of similar size. Therefore, meiotic segregation was used to separate these YACs; A80-C7 was crossed to YPH857 and a meiotic product was obtained which contained only the IgK YAC (MP8-2; host genotype = α ade2 leu2 his3 his5 lys2 ura3 trp1 can1 cyh2). The A80-C7 and A276-F2 YACs have been targeted with pLUTO to incorporate the human HPRT minigene into the YAC right vector arm.

Restriction analysis of the IgK YACs A80-C7 and A276-F2 using a number of enzymes supports the conclusion that both YACs are unrearranged (i.e., in germline configuration). For example, BamHI digestion followed by hybridization with the CK probe demonstrates the expected 13 kb restriction fragment (Klobeck et al., Biol. Chem. Hoppe-Seyler 370:1007-1012 (1989)). The same-sized band hybridizes to a JK probe (a 1.2 kb PCR product using primer set to amplify the JK1-5 region), as predicted from the genomic map (Klobeck et al., supra). The B3 class IV gene (probe is a 123 bp PCR product from the B3 gene) gives a 4.9 kb BamHI and a 2.2 kb BglII fragment, close to the published values of 4.6 kb and 2.3 kb, respectively

(Lorenz et al., Molec. Immunol. 25:479-484 (1988)). PCR analysis of both IgK YACs as well as human genomic DNA for the following kappa locus sequences revealed the predicted band sizes: Kde (120 bp), CK (304 bp), C-J intronic enhancer (455 bp), JK1-5 (1204 bp), B3 VK (123 bp) and B1 VK pseudogene (214 bp). Sequences used to design PCR primers for the CK, JK and C-J enhancer regions are from Whitehurst et al., Nucl. Acids. Res. 20:4929-4930 (1992); Kde is from Klobeck and Zachau, Nucl. Acids. Res. 14:4591-4603 (1986); B3 is from Klobeck et al., Nucl. Acids. Res. 13:6515-6529 (1985); and B1 is from Lorenz et al., supra.

B. Introduction of 680 kb yHPRT YAC into ES Cells

1. Culture of yHPRT yeast strain and preparation of yeast spheroplasts

The 680 kb yHPRT is a YAC containing a functional copy of the human hypoxanthine phosphoribosyltransferase (HPRT) gene cloned from a YAC library, as described in Huxley, et al. (1991) Genomics 9:742-750. The yeast strain containing the yHPRT was grown in uracil and tryptophan deficient liquid media, as described in Huxley, et al. (1991) supra.

To prepare the yeast spheroplasts, a 400 ml culture of yeast containing yHPRT was spun down and the yeast pellet was washed once with water and once with 1 M sorbitol. The yeast pellet was resuspended in SPEM (1 M sorbitol, 10 mM sodium phosphate pH 7.5, 10 mM EDTA pH 8.0, 30 mM β-mercaptoethanol) at a concentration of 5 x 10^8 yeast cells/ml. Zymolase 20T was added at a concentration of 150 μg/ml of yeast cells, and the culture was incubated at 30°C until 90% of the cells were spheroplasts (usually for 15-20 minutes). The cells were washed twice in STC (1 M sorbitol, 10 mM Tris pH 7.5, 10 mM $CaCl_2$) and resuspended in STC at a concentration of 2.5 x 10^8/ml.

2. Culture of E14TG2a ES Cells

HPRT-negative ES cell line E14TG2a was cultured as previously described.

3. Fusion of ES Cells and Yeast Spheroplasts

Exponentially growing E14TG2a ES cells growing on gelatin-coated dishes were trypsinized and washed three times with serum-free DMEM. A pellet of $2.5x10^8$ yeast spheroplasts was carefully overlaid with $5x10^6$ ES cells which were spun down onto the yeast pellet. The combined pellet was resuspended in 0.5 ml of either 50% polyethylene glycol (PEG) 1500 or 50% PEG 4000 (Boeringer Mannheim) containing 10 mM CaCl2. After 1.5 minutes incubation at room temperature or at 37°C, 5 ml of serum-free DMEM were added slowly, and the cells were left at room temperature for 30 minutes. The cells were then pelleted and resuspended in 10 ml of ES cell complete medium (as previously described) and were plated onto one 100 mm plate coated with feeder cells. After 24 hours the medium was replaced with fresh medium. Forty-eight hours post-fusion, HAT (ES media containing $1x10^{-4}$ M hypoxanthine, $4x10^{-7}$ M aminopterin, $1.6x10^{-5}$ thymidine) selection was imposed. HAT-resistant ES colonies were observed 7-10 days post-fusion in the plates from both the different fusion conditions used. yHPRT-ES ("ESY") fusion colonies were picked and plated onto feeder-coated wells, and expanded for further analysis.

4. Analysis of YAC DNA Integrated into yHPRT-ES Fusion Clones

DNA extracted form 23 yHPRT-ES fusion colonies was digested with HindIII and subjected to Southern blot analysis (Figure 12) using the probes: a human repetitive Alu sequence (A); pBR322-specific sequences for the right (B) and left (C) YAC vector arms; yeast Ty repetitive sequence (D); yeast single copy gene LYS2 (E). The human HPRT probe, a 1.6 kb full length cDNA (Jolly et al., Proc.

Natl. Acad. Sci. USA 80:477-481 (1983)) was used to confirm the presence of the human HPRT gene in ESY clones. The Alu probe was a 300 bp BamHI fragment from the BLUR8 Alu element in pBP63A (Pavan et al., Proc. Natl. Acad. Sci. USA 78:1300-1304 (1990)). The right and left vector arm probes were pBR322-derived BamHI-PvuII 1.7 and 2.7 kb fragments, respectively, which correspond to the vector sequences in pYAC4 (scheme a, b (Burke et al., in: Guide to Yeast Genetics and Molecular Biology, Methods in Enzymology, Guthrie and Fink, eds., Academic Press, 194:251-270 (1991)). The 4.5 kb fragment, detected by the right arm probe, spans the region between the HindIII site at the telomere 5′ end and the first HindIII site within the human insert (scheme a). The 3 kb and 4.1 kb fragments detected by the left end probe correspond to the region between the HindIII site at the telomere end and the HindIII site 5′ of the yeast sequences, and the region spanning from the HindIII site 3′ of the centromere into the human insert, respectively (scheme b). The difference in the hybridization intensity of these two bands relates to the difference in the amount of homology between these fragments and the probe. The yeast Ty repetitive probe (Philippsen et al., in Gene Expression in Yeast, Proceedings of the Alko Yeast Symposium, Helsinki, Korhola and Vaisanen, eds., Foundation for Biotechnical and Industrial Fermentation Research, 1:189-200 (1983)) was a 5.6 kb XhoI fragment isolated from Ty1-containing pJEF742 which could also detect the 3′ HindIII fragment of Ty2, due to the homology between the two elements. The LYS2 gene probe was a 1.7 BamHI fragment from pLUS (Hermanson et al., Nuc. Acids. Res. 19:4943-4948 (1991)).

Hybridization with a human HPRT probe (full length 1.6 kb cDNA probe) demonstrated that all the clones analyzed contained the same 15, 7 and 5 kb exon-containing fragments of the human HPRT gene as the yHPRT YAC. Reprobing the same blots with a human repetitive Alu

sequence 300 bp probe indicated that all the clones analyzed contained most, if not all, the Alu-containing fragments present in yHPRT (Figure 12A). These data indicate that in most of the clones analyzed the 680 kb human insert had not been detectably rearranged or deleted upon integration into the ES cell genome. Integration of YAC vector sequences was examined using probes specific for the vector arms. Rehybridization of the same blots with a probe for the right YAC vector arm, detecting a 4.5 kb HindIII fragment, indicated that in 10 out of 23 of the clones analyzed, the right YAC arm up to the telomere was still intact and unrearranged and linked to the human insert (Figure 12B) thus providing further evidence for the integrity of the YAC in these clones. The left arm probe detected the 3 kb and 4.1 kb HindIII yHPRT fragments in 18 out of the 20 clones analyzed (Figure 12C), indicating a high frequency of left arm retention.

The structural integrity of yHPRT in ESY clones was further evaluated for two clones (ESY 5-2 and 8-7) using pulsed-field gel restriction analysis. In yeast carrying yHPRT, five Sfi fragments of the following approximate sizes were defined by different probes: 315 kb (Alu, left arm), 145 kb (Alu, HPRT); 95 kb (Alu, right arm), 70 and 50 kb (Alu only). In both ES clones, the internal HPRT and Alu-specific fragments were similar in size to the yHPRT fragments. The end fragments detected for both clones were larger than those in yHPRT, as expected for YACs integrated within a mouse chromosome: 185 and 200 kb for the right end fragment, respectively, and over 800 kb for the left end fragment for both clones. These data, together with the Alu profile, provide additional evidence for the retention of the structural integrity of the YAC in these clones. These studies were complemented by fluorescence in-situ hybridization carried out on ESY 8-7 (Figure 13 A, B) and ESY 8-6 metaphase chromosome spreads in which a single integration site was detected for the

human sequences. Photomicrographs of representative metaphase spreads (Figure 13 A, B, C) or interphase nuclei (Figure 13D) from ESY 8-7 cells (Figure 13 A, B) hybridized with biotinylated human genomic sequences and ESY 8-6 cells (Figure 13 C, D) hybridized with biotinylated yeast repeated DNA sequences. The human probe was generated from human genomic placental DNA (Clontech, Palo Alto, CA). The yeast probe consisted of a mix of DNA fragments encoding the yeast repeated elements; delta (a 1.08 kb Sau3A fragment of pdelta6 (Gafner et al., EMBO J. 2:583-591 (1983)) and Ty (a 1.35 kb EcoRI-SaII fragment of p29 (Hermanson et al., Nuc. Acids. Res. 19:4943-4948 (1991)), the rDNAs (a 4.6 kb BgIIIk-A L90 and a 4.4 kb BgIII-B L92 fragment (Keil and Roeder, Cell 39:377-386 (1984)), and the Y' telomere elements (2.0 and 1.5 kb BgIII-HindIII fragments of p198 (Chan and Tye, Cell 33:563-573 (1983)). Hybridization of sequences on chromosome metaphase spreads with biotinylated probes and detection by Avidin-FITC followed by biotin-anti-Avidin and Avidin-FITC amplification was carried as described by Trask and Pinkel, Methods Cell Biol. 30:383-400 (1990), using a Zeiss Axiophot microscope. Chromosomes were counterstained with propidium iodide. The photomicrographs shown are representative of 95% of the metaphase spreads or interphase nuclei scanned in three independent experiments carried out with the human or the yeast probes. A single integration site was detected for the human sequences.

The same blots were also probed with the yeast Ty repetitive element sequence to detect the presence of yeast genomic DNA sequences in the ESY clones (Figure 12 D). Whereas some of the clones were found to contain most of the Ty-containing fragments present in the parental yeast strain, some of the clones were found to have a very small fraction, if at all, of the Ty-containing fragments. These results indicate that in some ES clones, although the YAC DNA is integrated intact, little or no yeast

genomic DNA was integrated. To determine if the yeast chromosomal DNA was integrated at single or multiple sites within the ES cell genome, fluorescent in-situ hybridization was performed on ESY clone 8-6 which had a complete Ty profile. A single integration site was detected using a combined yeast repetitive probe (Figure 13 C, D), indicating that within the limits of resolution, all yeast DNA fragments integrated in one block.

Using the ability of ES cells to undergo in vitro orderly differentiation, YAC stability and the effect of integrated DNA on the pluripotency of ES cells was investigated. Four ES clones, containing different amounts of yeast DNA (ESY 5-2, 3-6, 8-6 and 8-7) exhibited a differentiation pattern indistinguishable from that of unfused ES cells: formation of embryoid bodies giving rise to a variety of differentiated cell types (Figure 14 A). Southern blot analysis was performed on DNA extracted from differentiated ESY 5-2, 3-6, 8-5 and 8-6 (20 μg) and yHPRT in AB1380 (40 ng) using (a) a human Alu probe; (b) yeast Ty sequences. ES clones were induced to form embryoid bodies by culturing them as aggregates in suspension for 10-14 days as described by Martin and Evans, Cell 6:467-474 (1975). Following their reattachment to tissue culture substratum, ESY-derived embryoid bodies gave rise to differentiated cell types. YAC and yeast DNA sequences were stably retained by the differentiated ES clones during 40 days of culture in non-selective medium, demonstrating that the stably integrated foreign DNA did not impair the pluripotency of the ES cells (Figure 14 B). The differentiated cultures maintained a functional human HPRT gene as evidenced by their normal growth and differentiation when transferred to HAT-selective medium.

5. Generation of chimeric mice from yHPRT-ES cell lines

The ability of ESY cells to repopulate mice, including the germline, was demonstrated by microinjection of ES cells into mouse blastocysts and the generation of

chimeric mice. ESY cells were microinjected into C57BL/6J mouse blastocysts, and chimeric mice were generated as previously described. Chimeric males were mated with C57BL/6J females and germline transmission was determined by the presence of agouti offspring. Genomic DNA prepared from the tails of the chimeric mice were analyzed for the presence of the yHPRT DNA in the mouse genome by PCR analysis. The presence of the YAC left arm was analyzed using the two priming oligonucleotides, 5' TTCTCGGAGCACTGTC CGACC and 5' CTTGCGCCTTAAACCAACTTGGTACCG, which were derived, respectively, from the pBR322 sequences and the SUP4 gene within the YAC left vector arm. A 259 bp PCR product was obtained from the analysis of the yeast containing yHPRT and the ESY cell lines. PCR analysis of tail DNA prepared from 18 chimeric mice generated from ESY cell lines ESY3-1 ESY3-6 and ESY5-2, gave rise to the expected PCR product, thus indicating the presence of the YAC left vector arm in the genome of the chimeric mice.

6. Germline transmission of yHPRT

Chimeric males, with coat color chimerism of 30-60%, derived from the ESY cell lines ESY3-1 and ESY5-2 were set up for mating for germline transmission evaluation, i.e. to determine whether the genetic modification was passed via the germ cells (sperm or oocytes) to the progeny of the animals. Three of the chimeric ESY3-1 derived males, 394/95-1, 394/95-2 and 411-1 transmitted the ES cell genome to their offspring at a frequency of 20%, 30% and 30%, respectively. Southern blot analysis of tail DNA from the agouti pups indicated the presence of the yHPRT in the genome of three mice, 4-2, 4-3 and 5-1, derived from the 394/395-2 chimera. The Alu profile obtained from such analysis was indistinguishable from that of the parent ES3-1 cell line (Figure 14 C), demonstrating that the 680 kb human insert was transmitted faithfully through the mouse germline.

Using a human HPRT-specific PCR assay on mRNA-derived cDNAs from a yHPRT-containing offspring, the expression of the human HPRT gene in all the tissues tested was detected (Figure 15 A and B), thus demonstrating the transmitted YAC retained its function with fidelity. In this experiment, human HPRT mRNA was detected by reverse transcription (RT)-PCR in ES, ESY 3-1 and Hut 78 (human) cells, spleen and liver from a control mouse (C) or the 4-3 agouti offspring (derived from the 394/95-2 chimera) and a sample containing no template DNA (indicated as "-" in Figure 15A). Reverse transcription of poly (A+) RNA and PCR amplification of specific cDNA sequences were performed using the cDNA Cycle Kit (Invitrogen). Specific amplification of a 626 bp fragment from human HPRT cDNA in the presence of murine HPRT cDNA was performed as outlined by Huxley et al, supra. Integrity of all RNA samples was demonstrated by PCR amplification of cDNAs for the mouse γ-interferon receptor. The primers used to amplify a 359 bp fragment were: GTATGTGGAGCATAACCGGAG and CAGGTTTTGTCTCTAACGTGG. The human HPRT and the γ-interferon receptor primers were designed to eliminate the possibility of obtaining PCR products from genomic DNA contamination. PCR products were analyzed by electrophoresis and visualized with ethidium bromide. The size markers are 1 kb ladder (BRL). The results of detection of mouse γ-interferon receptor mRNA by RT-PCR in the samples described above are shown in Figure 15B. The specific human HPRT mRNA was also detected in the other tissues tested (brain, kidney and heart) derived from the 4-3 mouse. Comparable steady-state levels of mouse and human HPRT mRNA were detected in the liver of yHPRT-containing progeny. These results indicate that the uptake of as much as 13 megabases of yeast genomic DNA was not detrimental to proper development, germline transmission or gene expression.

The above results demonstrate that yeast spheroplasts are an effective vehicle for the delivery of a single copy

large molecular weight DNA fragment into ES cells and that such molecules are stably and functionally transmitted through the mouse germline. The Alu profiles, complemented by PFGE analysis and in situ hybridization for some of the ES clones, strongly argue that the majority of the clones contained virtually all the human insert in unrearranged form (i.e. in "germline configuration"), with a high frequency of clones (40%) also retaining both YAC arms. The significant uptake of yeast genomic DNA was not detrimental to proper differentiation of ES cells in vitro and in vivo and did not prevent germline transmission or gene expression. By these methods, one can transmit large fragments of genomic DNA as inserts into non-human animal genomes, where the inserts may be transmitted intact by germline transmission. Therefore, a wide variety of xenogeneic DNA can be introduced into non-human hosts such as mammals, particularly small laboratory animals, that may impart novel phenotypes or novel genotypes. For example, one can provide in small laboratory animals genes of a mammal, such as a human, to study the etiology of a disease, the response to human genes to a wide variety of agents. Alternatively, one can introduce large loci into a mammalian host to produce products of other species, for example humans, to provide human protein sequences of proteins such as immunoglobulins, T-cell receptors, major histocompatibility complex antigens, etc.

Introduction of heavy chain YAC A287-C10 and kappa chain YAC A80-C
into ES cells and Embryos

Yeast containing the human heavy chain YAC A287-C10 targeted with pLUTO (yA287-C10) were spheroplasted and fused with the HPRT-deficient ES cell line E14.1TG3B1 as described above. Ten HAT-resistant ES (ESY) clones (2B, 2C, 2D, 3A, 3B, 5C, 1125A, 1125E, 100/1500 and 100/4000) were picked and were expanded for DNA analysis.

Evaluation of the integrated YAC was performed by Southern blot analysis of HindIII-digested DNA from these clones, using human heavy chain probes for the D, J_H, μ, and VH2 regions, decribed above. All ESY clones were found to contain the expected > 10 kb J_H and μ fragments. All ESY clones except 2D and 5C clones, were found to contain the 4.8 kb VH2 kb fragment. All ESY clones, except 2D and 3B were found to contain the expected 10 and 7.6 kb D gene fragments. Yeast genomic sequences were detected by hybridization to the yeast repetitive Ty element in all ESY clones except 2B, 2D, 100/1500 and 5C. ESY clones 2B, 3A and 5C were microinjected into C57B/6 blastocysts as described above and chimeric mice (10 from 2B clone, 1 from 3A clone and 1 from 5C clone) were generated. Southern blot analysis of tail DNA from 10 of these chimeric animals, indicated the presence of most, if not all, of the apparent 10 Alu fragments, detected in yA287-C10 in yeast, as well as the presence of VH_2 and D gene fragments. The generated chimeric mice were bred with C57BL16J mice for germline transmission evaluation. A chimeric male 78K-3 derived from the 2B clone transmitted the ES cell genome to its offspring at a frequency of 100%. Southern blot analysis of tail DNA from 4 out of 6 agouti mice pups indicated the presence of human heavy chain sequences.

Fusion experiments with yeast containing the human kappa chain YAC A80-C7 targeted with pLUTO (yA80-C7) with E14.1TG3B1 ES cells generated 2 HAT-resistant ESY clones: M4.4.1 and M5.2.1. Southern blot analysis of HindIII-digested DNAs from these clones revealed the presence of all the apparent 10 Alu fragments detected in yA80-C7 in yeast. In both clones yeast genomic sequences were integrated. ESY clones were microinjected into C57B1/6J blastocysts and chimeric mice were generated.

EXAMPLE VII

Production of human Ig by chimeric mice by Introduction of Human Ig using Homologous Recombination

As an alternative approach to that set forth in Examples I-VI, human Ig genes are introduced into the mouse Ig locus by replacing mouse heavy and light chain immunoglobulin loci directly with fragments of the human heavy and light chain loci using homologous recombination. This is followed by the generation of chimeric transgenic animals in which the embryonic stem-cell derived cells contribute to the germ line.

A. Construction of human heavy chain replacement vector.

The replacing human sequences include the SpeI 100 kb fragment of genomic DNA which encompasses the human VH6-D-J-Cμ-Cδ heavy chain region isolated from a human-YAC library as described before. The flanking mouse heavy chain sequences, which drive the homologous recombination replacement event, contain a 10 kb BamHI fragment of the mouse Cε-Cα heavy chain and a 5′ J558 fragment comprising the 5′ half of the J558 fragment of the mouse heavy chain variable region, at the 3′ and 5′ ends of the human sequences, respectively (Figure 16). These mouse sequences are isolated from a mouse embryo genomic library using the probes described in Tucker et al. (1981), PNAS USA, 78: 7684-7688 and Blankenstein and Krawinkel (1987, supra), respectively. The 1150 bp XhoI to BamHI fragment, containing a neomycin-resistance gene driven by the Herpes simplex virus thymidine kinase gene (HSV-tk) promoter and a polyoma enhancer is isolated from pMC1Neo (Koller and Smithies, 1989, supra). A synthetic adaptor is added onto this fragment to convert the XhoI end into a BamHI end and the resulting fragment is joined to the BamHI mouse Cε-Cα in a plasmid.

From the YAC clone containing the human heavy chain locus, DNA sequences from each end of the insert are recovered either by inverse PCR (Silverman et al. (1989), PNAS, 86:7485-7489), or by plasmid rescue in E. coli,

(Burke et al., (1987); Garza et al. (1989) Science, 246:641-646; Traver et al., 1989) (see Figure 8). The isolated human sequence from the 5'V6 end of the YAC is ligated to the mouse J558 sequence in a plasmid and likewise, the human sequence derived from the 3'Cd end of the YAC is ligated to the Neo gene in the plasmid containing Neo and mouse Cε-Cα described above. The human V6-mouse J558 segment is now subcloned into a half-YAC cloning vector that includes a yeast selectable marker (HIS3) not present in the original IgH YAC, a centromere (CEN) and a single telomere (TEL). The human Cδ-Neo-mouse Cε-Cα is likewise subcloned into a separate half-YAC vector with a different yeast selectable marker (LEU2) and a single TEL. The half-YAC vector containing the human V6 DNA is linearized and used to transform a yeast strain that is deleted for the chromosomal HIS3 and LEU2 loci and which carries the IgH YAC. Selection for histidine-prototrophy gives rise to yeast colonies that have undergone homologous recombination between the human V6 DNA sequences and contain a recombinant YAC. The half-YAC vector containing the human Cδ DNA is then linearized and used to transform the yeast strain generated in the previous step. Selection for leucine-prototrophy results in a yeast strain containing the complete IgH replacement YAC (see Figure 16). Preferably, both targeting events are performed in a single transformation step, selecting simultaneously for leucine and histidine prototrophy. This is particularly useful when the original centric and acentric YAC arms are in opposite orientation to that shown in Figure 16. This YAC is isolated and introduced into ES cells by microinjection as described previously for embryos.

EXAMPLE VIII

Crossbreeding of transgenic mice

A. Generation of human monoclonal antibody producing mice

Mice containing the human immunoglobulin locus are mated to mice with inactivated murine immunoglobulin genes to generate mice that produce only human antibodies. Starting with four heterozygous strains, three generations of breeding are required to create a mouse that is homozygous for inactive murine kappa and heavy chain immunoglobulins, and heterozygous for human heavy and kappa chain immunoglobulin loci. The breeding scheme is shown in Figure 17.

EXAMPLE IX

Production of Human Monoclonal Antibodies

A. Immunization of mice

Germline chimeric mice containing integrated human DNA from the immunoglobulin loci are immunized by injection of an antigen in adjuvant. The mice are boosted with antigen 14 days after the primary immunization, repeated after 35 and 56 days. A bleed is done on the immunized animals to test the titer of serum antibodies against the immunizing antigen. The mouse with the highest titer is sacrificed, and the spleen removed.

B. Fusion of splenocytes

Myeloma cells used as the fusion partner for the spleen cells are thawed 6 days prior to the fusion, and grown in tissue culture. One day before the fusion, the cells are split into fresh medium containing 10% fetal calf serum at a concentration of 5×10^5 cells/ml. On the morning of the fusion the cells are diluted with an equal volume of medium supplemented with 20% fetal calf serum and 2X OPI (3 mg/ml oxaloacetate, 0.1 mg/ml sodium pyruvate and 0.4 IU/ml insulin) solution.

After sacrificing the mouse, the spleen is aseptically removed, and placed in a dish with culture medium. The cells are teased apart until the spleen is torn into fine pieces and most cells have been removed.

The cells are washed in fresh sterile medium, and the clumps allowed to settle out.

The splenocytes are further washed twice by centrifugation in medium without serum. During the second wash, the myeloma cells are also washed in a separate tube. After the final wash the two cell pellets are combined, and centrifuged once together.

A solution of 50% polyethylene glycol (PEG) is slowly added to the cell pellet while the cells are resuspended, for a total of two minutes. 10 ml of prewarmed medium is added to the cell solution, stirring slowly for 3 minutes. The cells are centrifuged and the supernatant removed. The cells are resuspended in 10 ml of medium supplemented with 20% fetal calf serum, 1X OPI solution and 1X AH solution (58 μM azaserine, 0.1 mM hypoxanthine). The fused cells are aliquoted into 96-well plates, and cultured at 37° for one week.

Supernatant is aseptically taken from each well, and put into pools. These pools are tested for reactivity against the immunizing antigen. Positive pools are further tested for individual wells. When a positive well has been identified, the cells are transferred from the 96-well plate to 0.5 ml of medium supplemented with 20% fetal calf serum, 1X OPI, and 1X AH in a 24-well plate. When that culture becomes dense, the cells are expanded into 5 ml, and then into 10 ml. At this stage the cells are sub-cloned so that a single antibody producing cell is in the culture.

In accordance with the above procedures, a chimeric non-human host, particularly a murine host, may be produced which can be immunized to produce human antibodies or analogs specific for an immunogen. In this manner, the problems associated with obtaining human monoclonal antibodies are avoided, because the transgenic host can be immunized with immunogens which could not be used with a human host. Furthermore, one can provide for booster injections and adjuvants which would not be

permitted with a human host. The resulting B-cells may then be used for immortalization for the continuous production of the desired antibody. The immortalized cells may be used for isolation of the genes encoding the immunoglobulin or analog and be subjected to further molecular modification by methods such as in-vitro mutagenesis or other techniques to modify the properties of the antibodies. These modified genes may then be returned to the immortalized cells by transfection to provide for a continuous mammalian cellular source of the desired antibodies. The subject invention provides for a convenient source of human antibodies, where the human antibodies are produced in analogous manner to the production of antibodies in a human host. The animal host cells conveniently provide for the activation and rearrangement of human DNA in the host cells for production of human antibodies.

In accordance with the subject invention, human antibodies can be produced to human immunogens, eg. proteins, by immunization of the subject host mammal with human immunogens. The resulting antisera will be specific for the human immunogen and may be harvested from the serum of the host. The immunized host B cells may be used for immortalization, eg. myeloma cell fusion, transfection, etc. to provide immortal cells, eg. hybridomas, to produce monoclonal antibodies. The antibodies, antiserum and monoclonal antibodies will be glycosylated in accordance with the species of the cell producing the antibodies. Rare variable regions of the Ig locus may be recruited in producing the antibodies, so that antibodies having rare variable regions may be obtained.

All publications and patent applications cited in this specification are herein incorporated by reference as if each individual publication or patent application were specifically and individually indicated to be incorporated by reference.

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be readily apparent to those of ordinary skill in the art in light of the teachings of this invention that certain changes and modifications may be made thereto without departing from the spirit or scope of the appended claims.

CLAIMS

WHAT IS CLAIMED IS:

1. An improved method for modifying the genome of a embryonic stem cell so as to contain, in the genome thereof, a xenogeneic DNA segment of at least 100 kb which method comprises:

combining under fusing conditions embryonic stem cells and yeast spheroplasts, said spheroplasts containing a yeast artificial chromosome (YAC) comprising said xenogeneic DNA segment and including a marker for selection, whereby said xenogeneic DNA segment becomes integrated into the genome of said embryonic stem cells; and

selecting for an embryonic stem cell carrying said xenogeneic DNA segment by means of the marker.

2. An improved method for producing a modified non-human animal, said animal having a xenogeneic DNA segment of at least 100 kb stably integrated into the genome of at least some cells of said animal, said method comprising:

combining under fusing conditions embryonic stem cells of said animal and yeast spheroplasts, said spheroplasts containing a yeast artificial chromosome (YAC) comprising said xenogeneic DNA segment and including a marker for selection, whereby said xenogeneic DNA segment becomes integrated into the genome of said embryonic stem cells;

selecting for embryonic stem cells carrying said xenogeneic DNA segment by means of the marker; and

transferring said selected embryonic cells into a host blastocyst, implanting said blastocyst in a pseudopregnant animal recipient, and allowing said blastocyst to develop to term to produce a chimeric animal carrying said xenogeneic DNA segment integrated into the genome of at least some cells of said animal.

3. An improved method for producing a modified non-human animal, said animal having a xenogeneic DNA segment of at least 100 kb stably integrated into the genome of said animal, said method comprising:

combining under fusing conditions embryonic stem cells of said animal and yeast spheroplasts, said spheroplasts containing a yeast artificial chromosome (YAC) comprising said xenogeneic DNA segment and including a marker for selection, whereby said xenogeneic DNA segment becomes integrated into the genome of said embryonic stem cells;

selecting for embryonic stem cells carrying said xenogeneic DNA segment by means of the marker;

transferring said selected embryonic cells into a host blastocyst and implanting said blastocyst in a pseudopregnant animal recipient, and allowing said blastocyst to develop to term to produce a chimeric animal carrying said xenogeneic DNA segment; and

mating said chimeric animal with an animal of the same species to produce said modified animal carrying said xenogeneic DNA segment.

4. A method according to any one of claims 1, 2 or 3, wherein said marker is the HPRT gene and said embryonic stem cells are HPRT deficient.

5. A method according to any of claims 1, 2 or 3, wherein said xenogeneic DNA is human DNA, and/or

wherein said xenogeneic DNA is immunoglobulin DNA in substantially intact form.

6. Embryonic stem cells comprising a genome modified according to the method of claims 1, 4 or 5.

7. A modified animal produced according to the method of claims 2, 3, 4 or 5.

8. The stem cells of claim 6 which are of a rodent.

9. The stem cells of claim 8 which are murine.

10. The animal of claim 7 which is a rodent.

11. The animal of claim 10 which is a mouse.

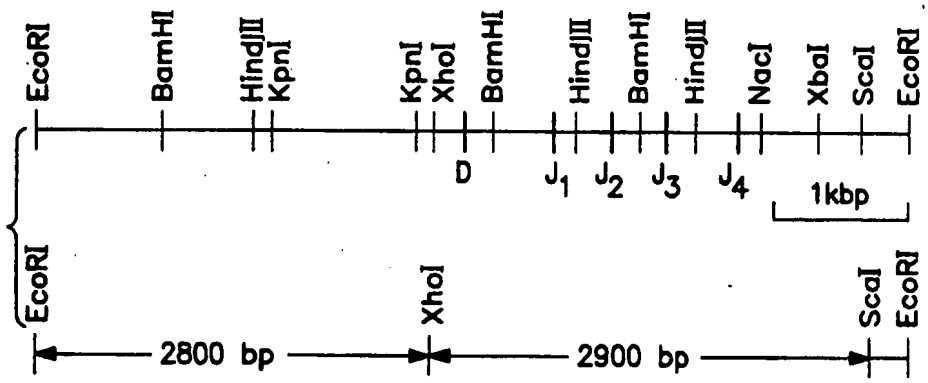

EcoRI
BamHI
HindIII
KpnI
KpnI
XhoI
BamHI
HindIII
BamHI
HindIII
NaeI
XbaI
ScaI
EcoRI
D
J1
J2
J3
J4
1kbp
EcoRI
XhoI
ScaI
EcoRI
2800 bp
2900 bp

FIG. IA

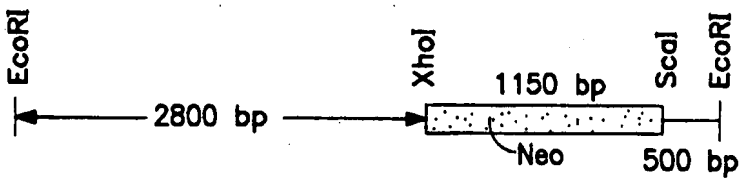

EcoRI
XhoI
1150 bp
ScaI
EcoRI
2800 bp
Neo
500 bp

FIG. IB

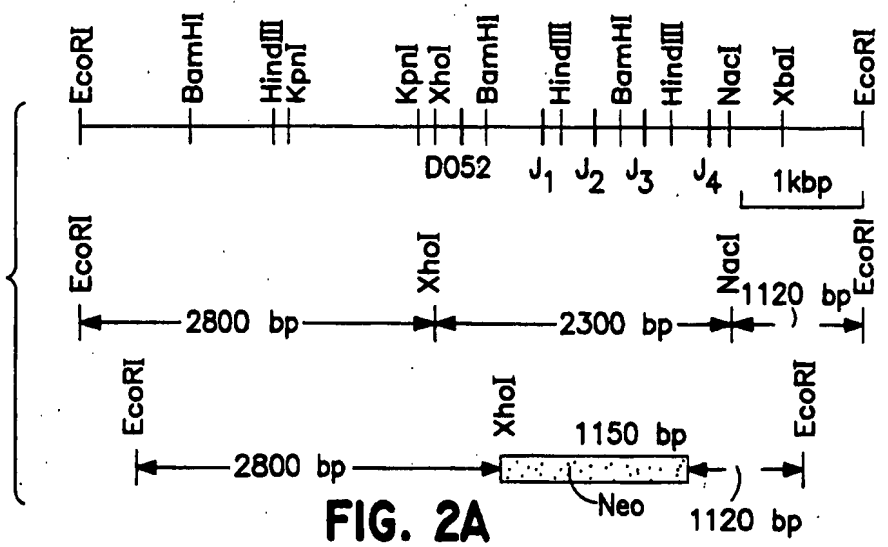


FIG. 2A

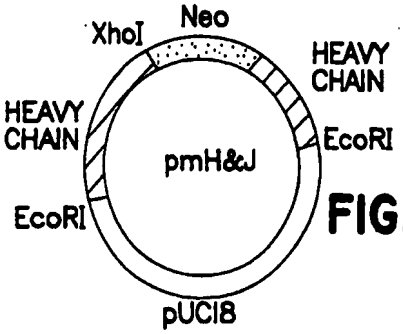


FIG. 2B

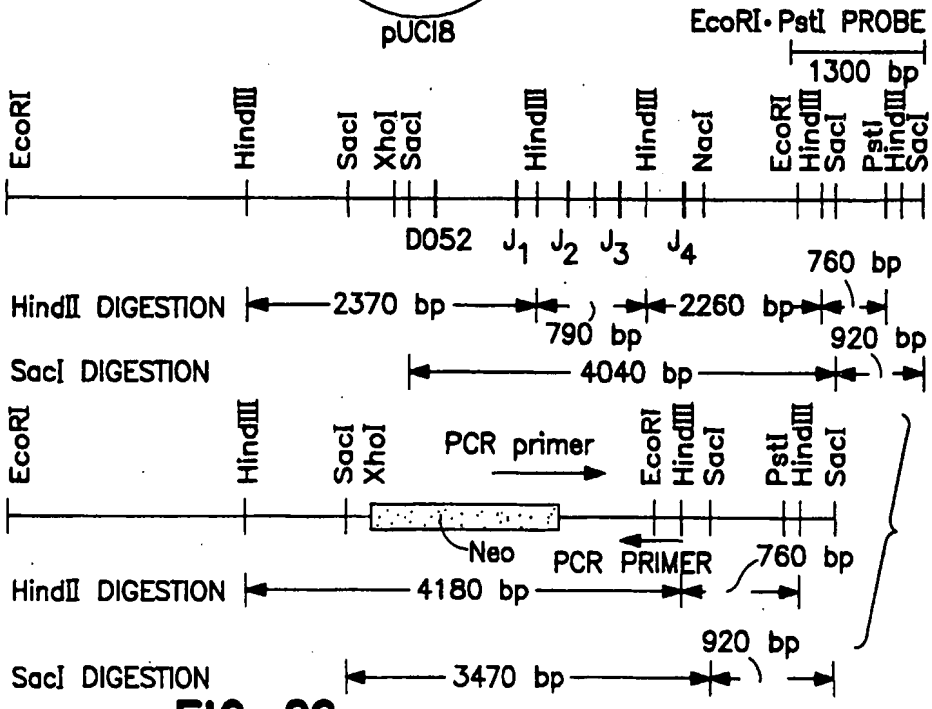


FIG. 2C

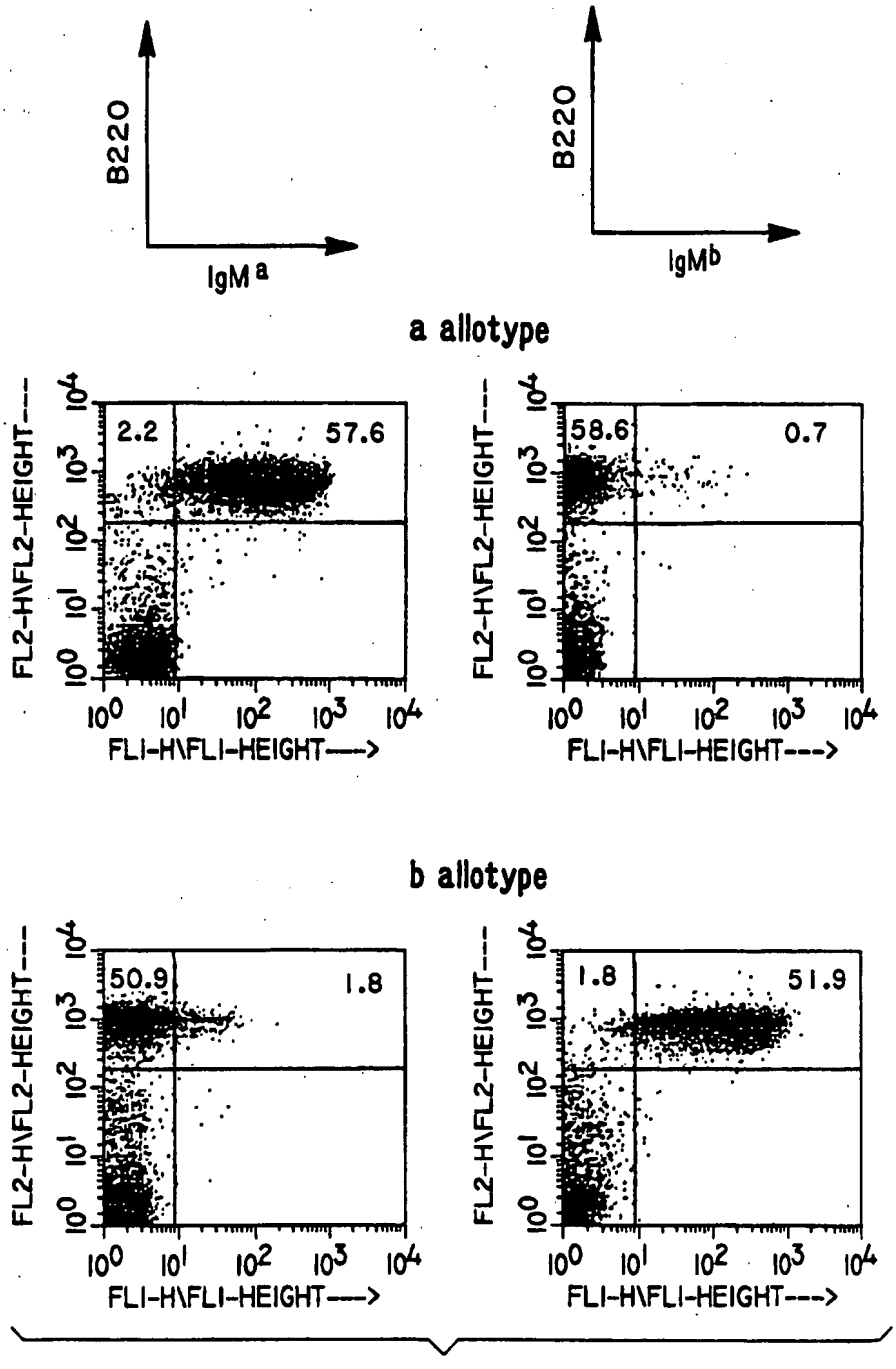

B220
IgM a
B220
IgMb
a allotype
FL2-H\FL2-HEIGHT---
FLI-H\FLI-HEIGHT--->
2.2
57.6
58.6
0.7
b allotype
50.9
1.8
1.8
51.9

FIG. 3-1

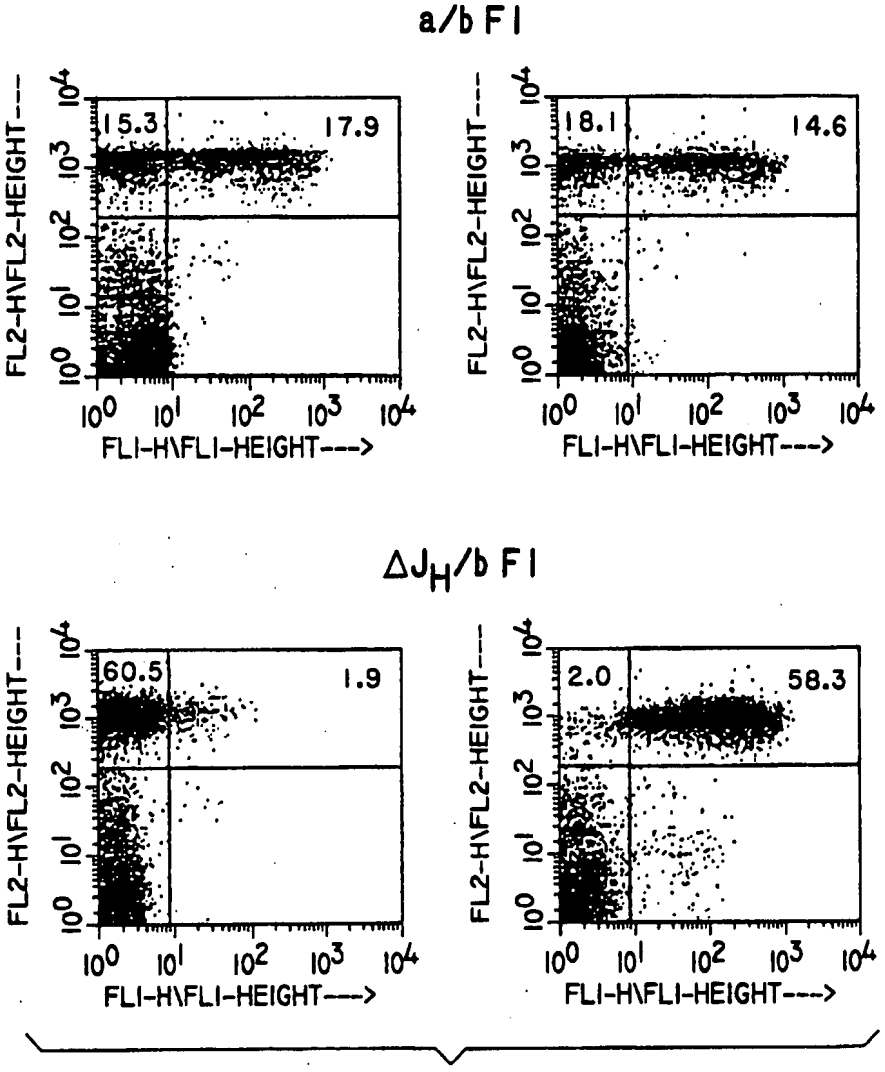

a/b F1
15.3
17.9
18.1
14.6
ΔJH/b F1
60.5
1.9
2.0
58.3
FL2-H\FL2-HEIGHT---
FLI-H\FLI-HEIGHT--->
10^0
10^1
10^2
10^3
10^4

FIG. 3-2

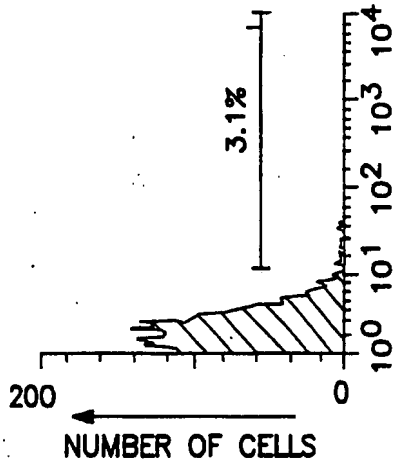


FIG. 4C

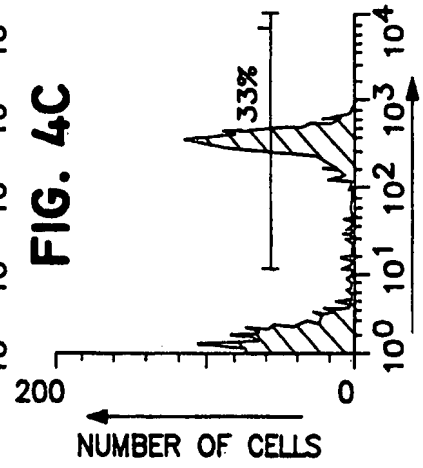


FIG. 4F

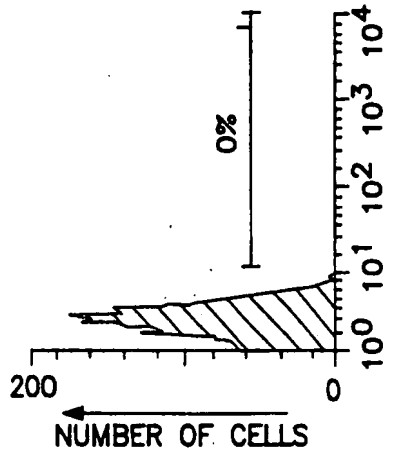


FIG. 4B

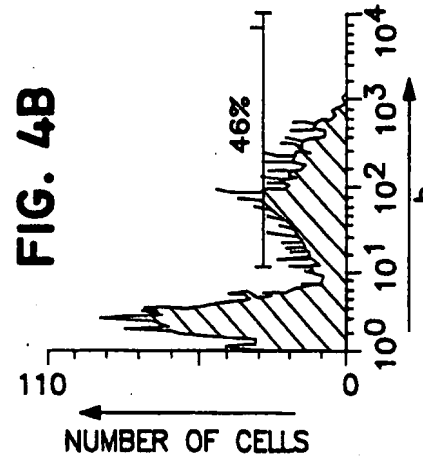


FIG. 4E

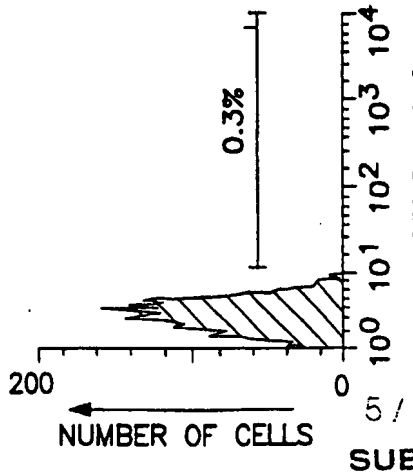


FIG. 4A

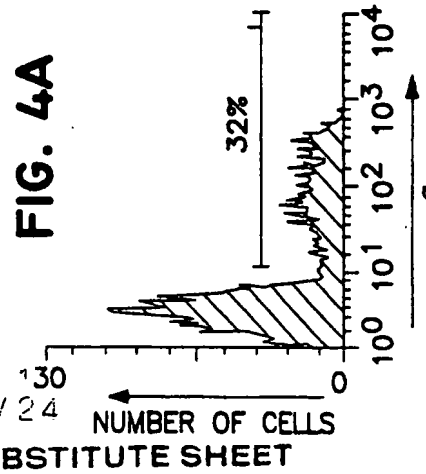


FIG. 4D

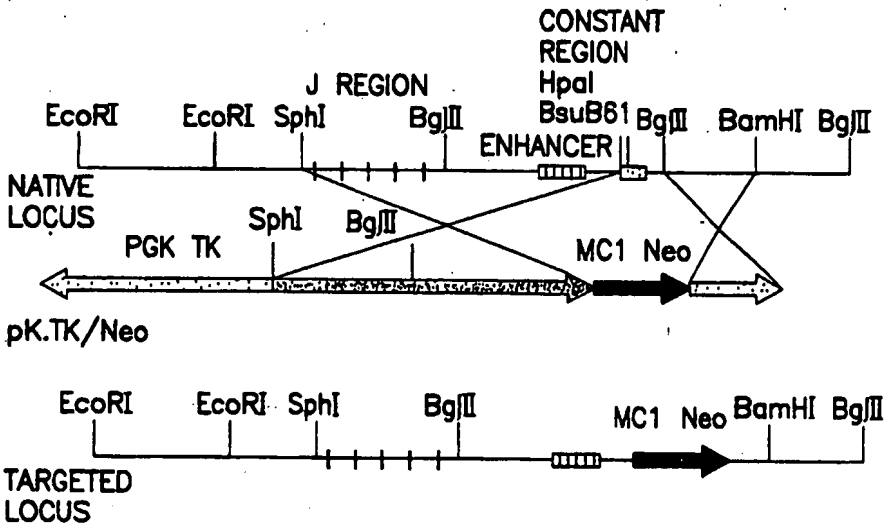
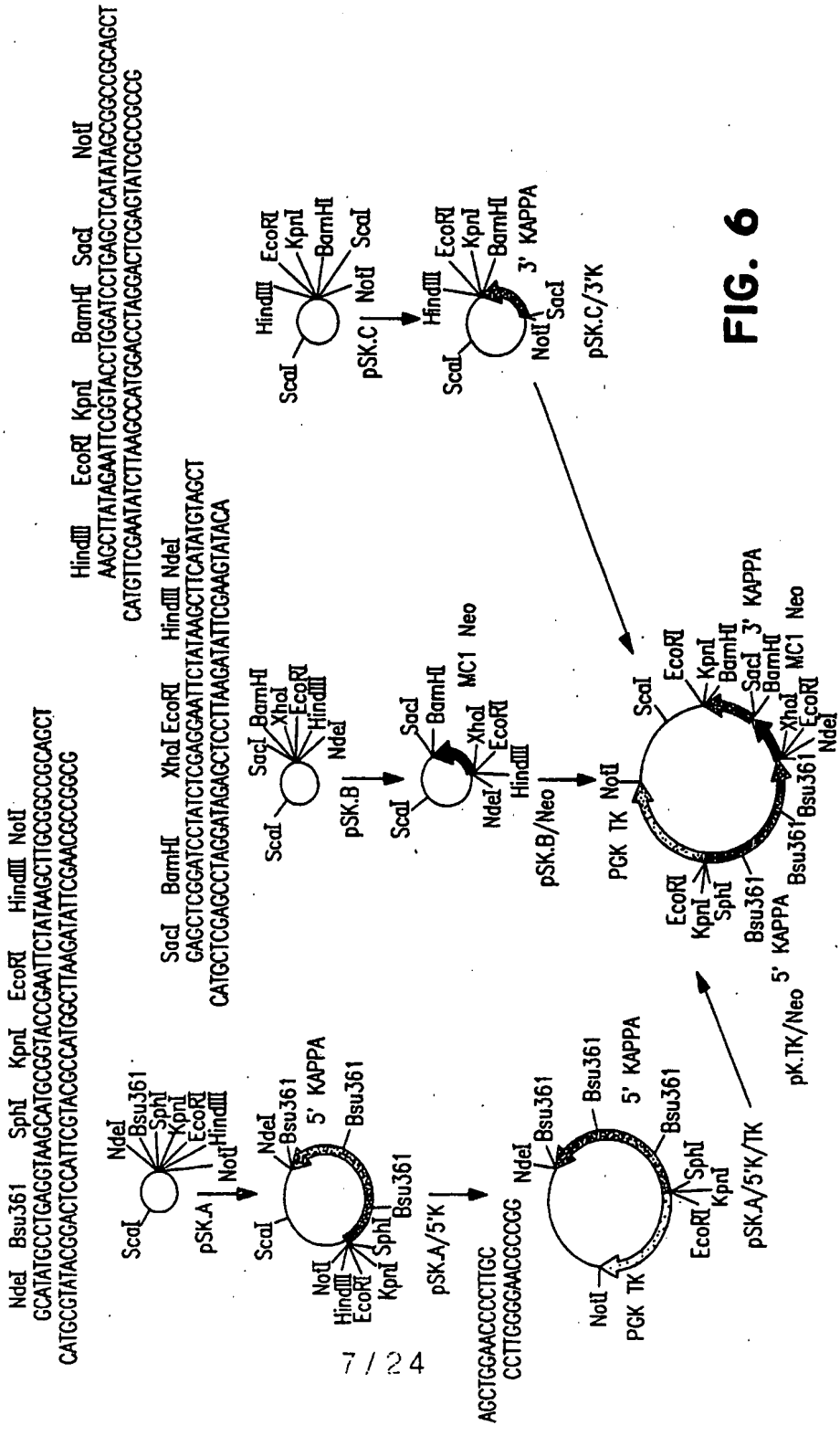

CONSTANT
REGION
HpaI
BsuB61
J REGION
EcoRI
EcoRI
SphI
BglII
ENHANCER
BglII
BamHI
BglII
NATIVE
LOCUS
SphI
BglII
PGK
TK
MC1
Neo
pK.TK/Neo
EcoRI
EcoRI
SphI
BglII
MC1
Neo
BamHI
BglII
TARGETED
LOCUS

FIG. 5


NdeI Bsu361 SphI KpnI EcoRI HindIII NotI
GCATATGCCTGAGGTAAGCATGCGGTACCGAATTCTATAAGCTTGCGGCCGCAGCT
CATGCGTATACGGACTCCATTCGTACGCCATGGCTTAAGATATTCGAACGCCGGCG
pSK.A
pSK.A/5'K
pSK.B
pSK.B/Neo
pSK.C
pSK.C/3'K
pK.TK/Neo
pSK.A/5'K/TK
5' KAPPA
3' KAPPA
PGK TK
MC1 Neo
SacI BamHI XhoI EcoRI HindIII NdeI
GAGCTCGGATCCTATCTCGAGCAATTCTATAAGCTTCATATGTAGCT
CATGCTCGAGCCTAGGATAGAGCTCGTTAAGATATTCGAAGTATACA
HindIII EcoRI KpnI BamHI SacI NotI
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CATGTTCGAATATCTTAAGCCATGGACCTAGGACTCGAGTATCGCCGCCG

FIG. 6

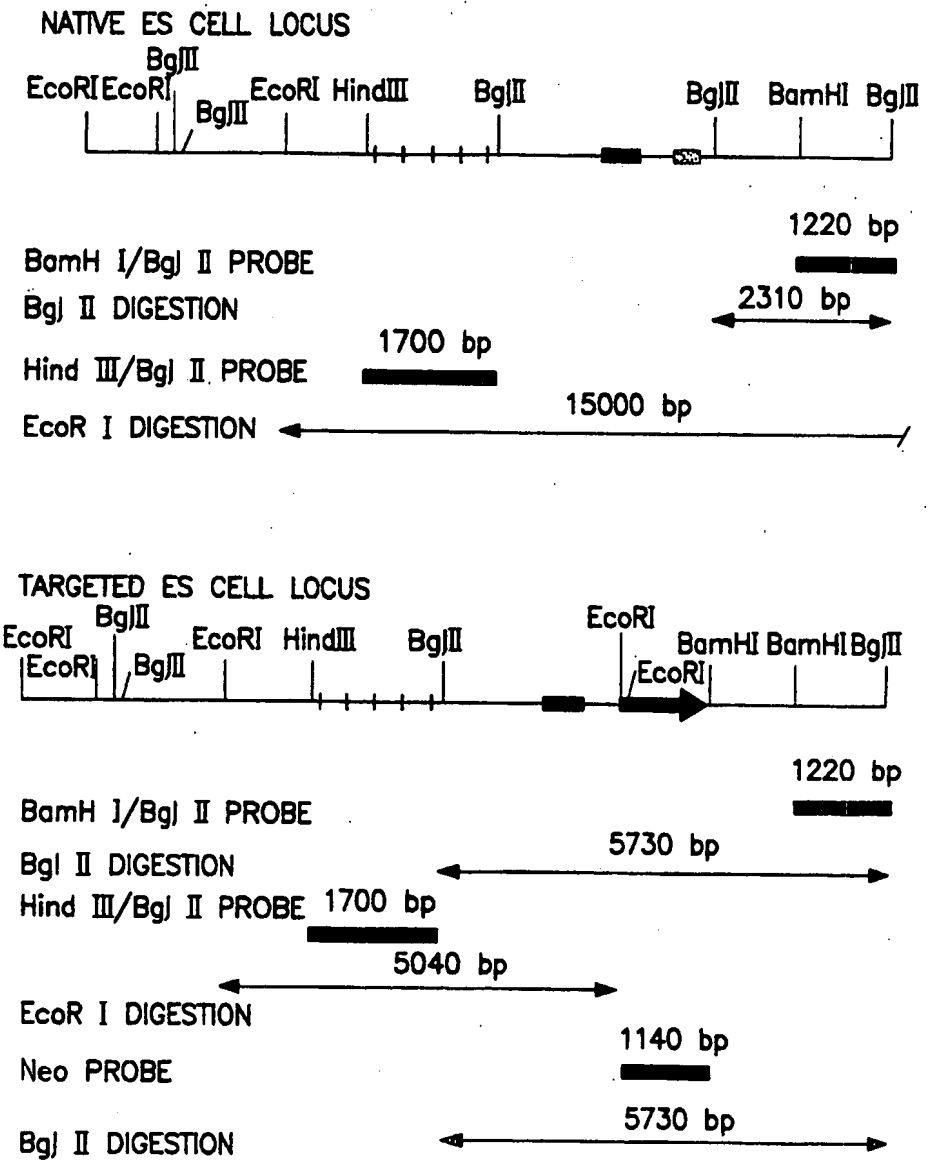

NATIVE ES CELL LOCUS
EcoRI EcoRI BglII EcoRI HindIII BglII BglII BamHI BglII
1220 bp
BamH I/Bgl II PROBE
Bgl II DIGESTION
2310 bp
1700 bp
Hind III/Bgl II PROBE
15000 bp
EcoR I DIGESTION
TARGETED ES CELL LOCUS
EcoRI BglII EcoRI HindIII BglII EcoRI BamHI BamHI BglII
EcoRI BglII EcoRI
1220 bp
BamH I/Bgl II PROBE
5730 bp
Bgl II DIGESTION
Hind III/Bgl II PROBE 1700 bp
5040 bp
EcoR I DIGESTION
1140 bp
Neo PROBE
5730 bp
Bgl II DIGESTION

FIG. 7

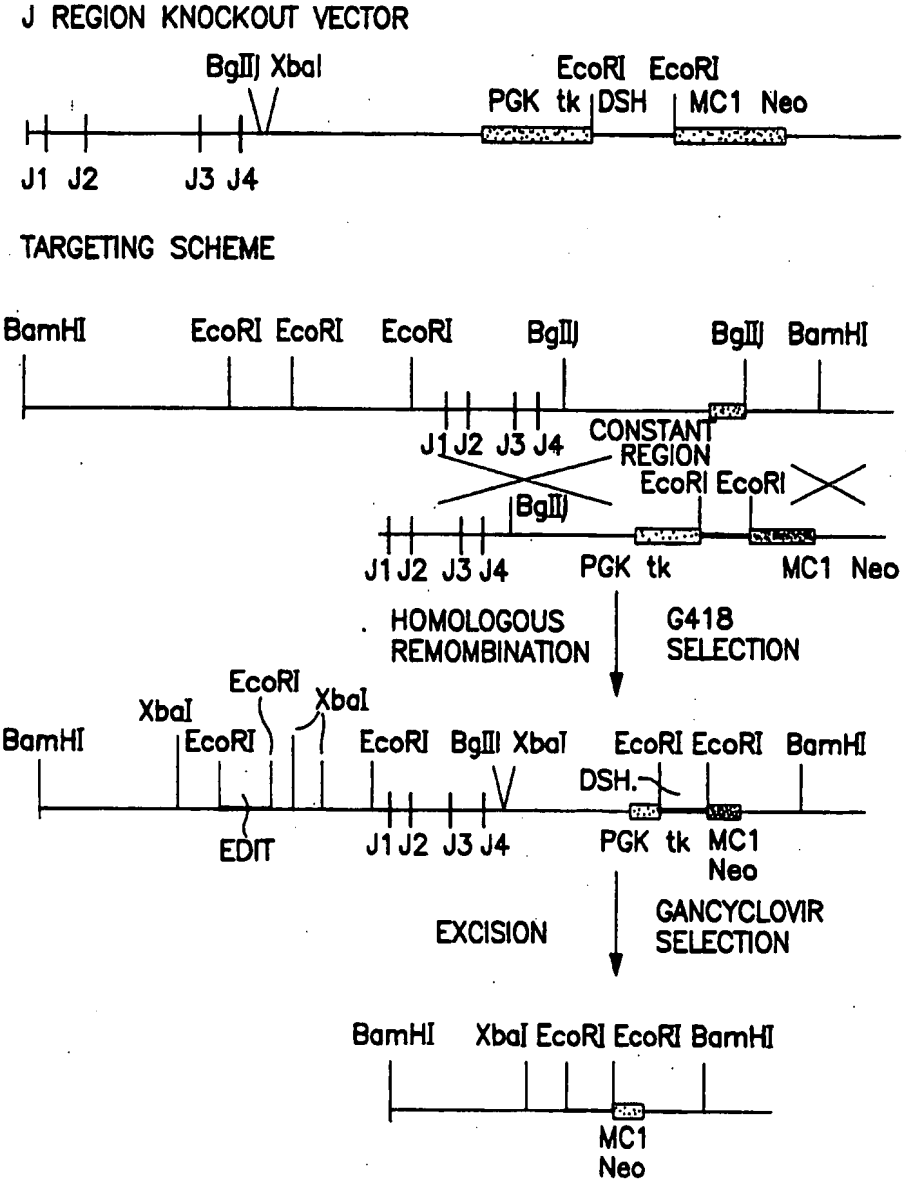

J REGION KNOCKOUT VECTOR
BglII XbaI
EcoRI EcoRI
PGK tk DSH MC1 Neo
J1 J2 J3 J4
TARGETING SCHEME
BamHI EcoRI EcoRI EcoRI BglII BglII BamHI
J1 J2 J3 J4
CONSTANT REGION
EcoRI EcoRI
BglII
J1 J2 J3 J4 PGK tk MC1 Neo
HOMOLOGOUS REMOMBINATION
G418 SELECTION
EcoRI
XbaI
XbaI
BamHI
EcoRI
EcoRI BglII XbaI
EcoRI EcoRI BamHI
DSH.
EDIT
J1 J2 J3 J4
PGK tk MC1 Neo
EXCISION
GANCYCLOVIR SELECTION
BamHI XbaI EcoRI EcoRI BamHI
MC1 Neo

FIG. 8

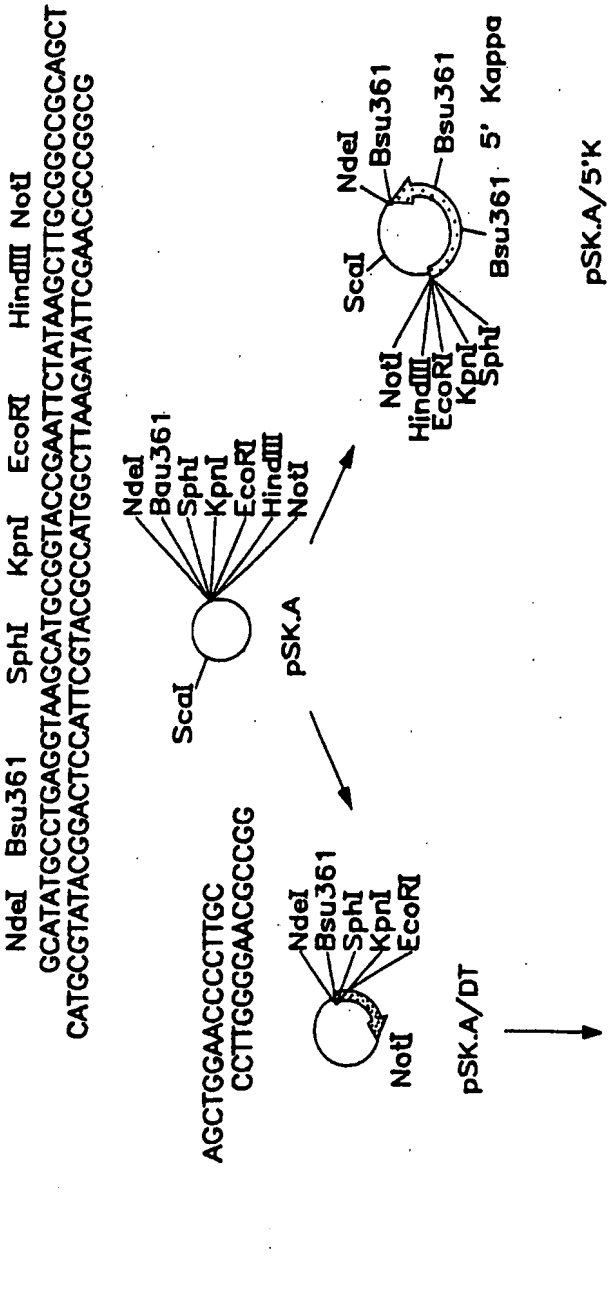

NdeI Bsu361 SphI KpnI EcoRI HindIII NotI
GCATATGCCTGAGGTAAGCATGCGGTACCGAATTCTATAAGCTTGCGGCCGCAGCT
CATGCGTATACGGACTCCATTCGTACGCCATGGCTTAAGATATTCGAACGCCGGCG
NdeI
Bsu361
SphI
KpnI
EcoRI
HindIII
NotI
ScaI
pSK.A
AGCTGGAACCCCTTGC
CCTTGGGGAACGCCGG
NdeI
Bsu361
SphI
KpnI
EcoRI
NotI
pSK.A/DT
NdeI
Bsu361
ScaI
NotI
HindIII
EcoRI
KpnI
SphI
Bsu361
Bsu361
5' Kappa
pSK.A/5'K
NdeI
Bsu361
Bsu361
5' Kappa
SphI
KpnI
NotI
EcoRI
PGK DT
p.SK.A/5'K/DT

FIG. 9-I

SacI BamHI XhoI EcoRI HindIII NdeI
GAGCTCGGATCCTATCTCGAGGAATTCTATAAGCTTCATATGTAGCT
CATCCTCGAGCCTAGGATAGAGCTCCTTAAGATATTCGAAGTATACA

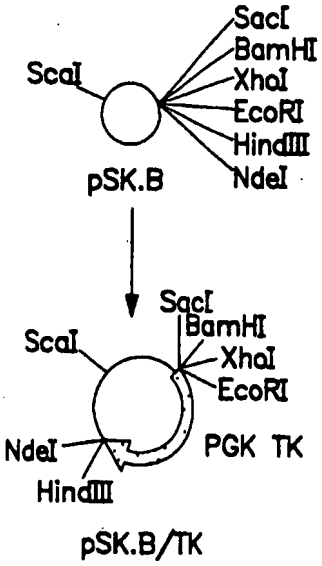


FIG. 9-2

HindIII EcoRI KpnI BamHI SacI NotI
AAGCTTATAGAATTCGGTACCTGGATCCTGAGCTCATAGCGGCCGCAGCT
CATGTTCGAATATCTTAAGCCATGGACCTAGGACTCGAGTATCGCCGGCG

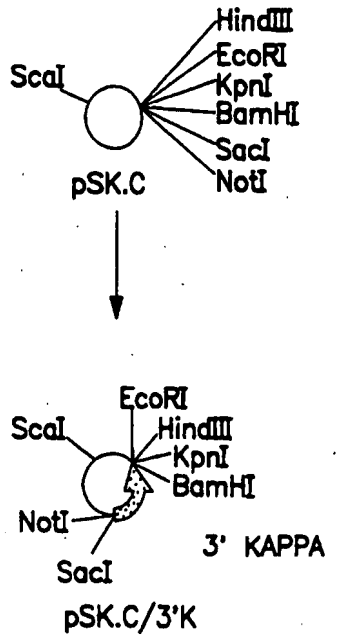
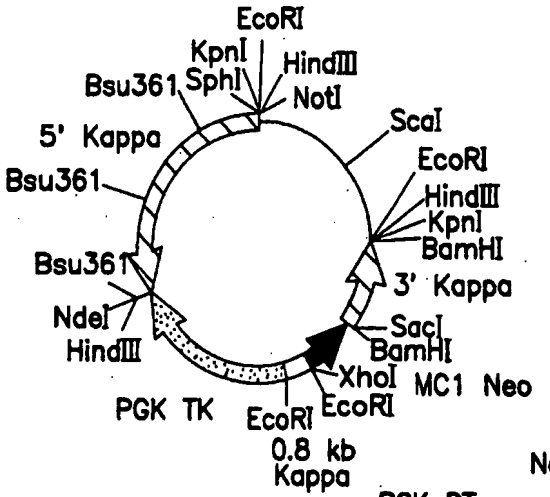


FIG. 9-3


EcoRI
KpnI
HindIII
Bsu361
SphI
NotI
5' Kappa
ScaI
Bsu361
EcoRI
HindIII
KpnI
BamHI
Bsu361
3' Kappa
NdeI
SacI
HindIII
BamHI
XhoI
MC1 Neo
PGK TK
EcoRI
EcoRI
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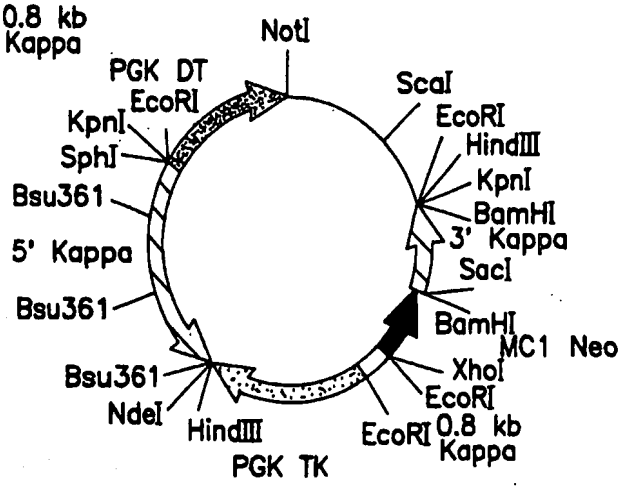
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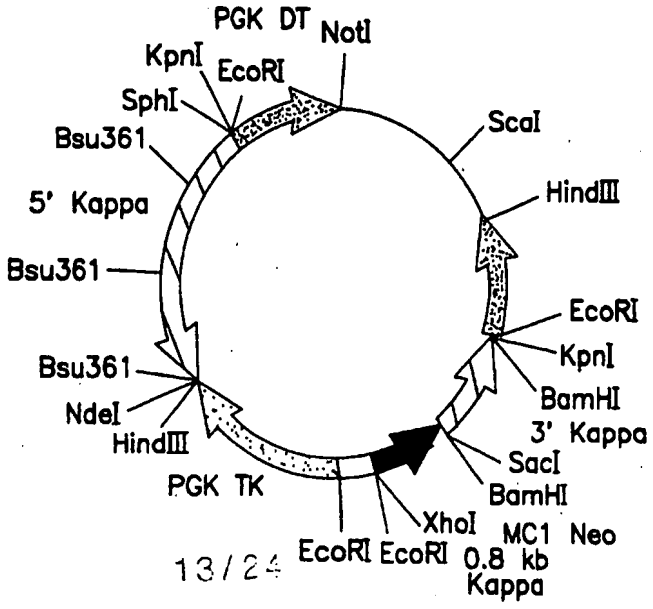
FIG. 10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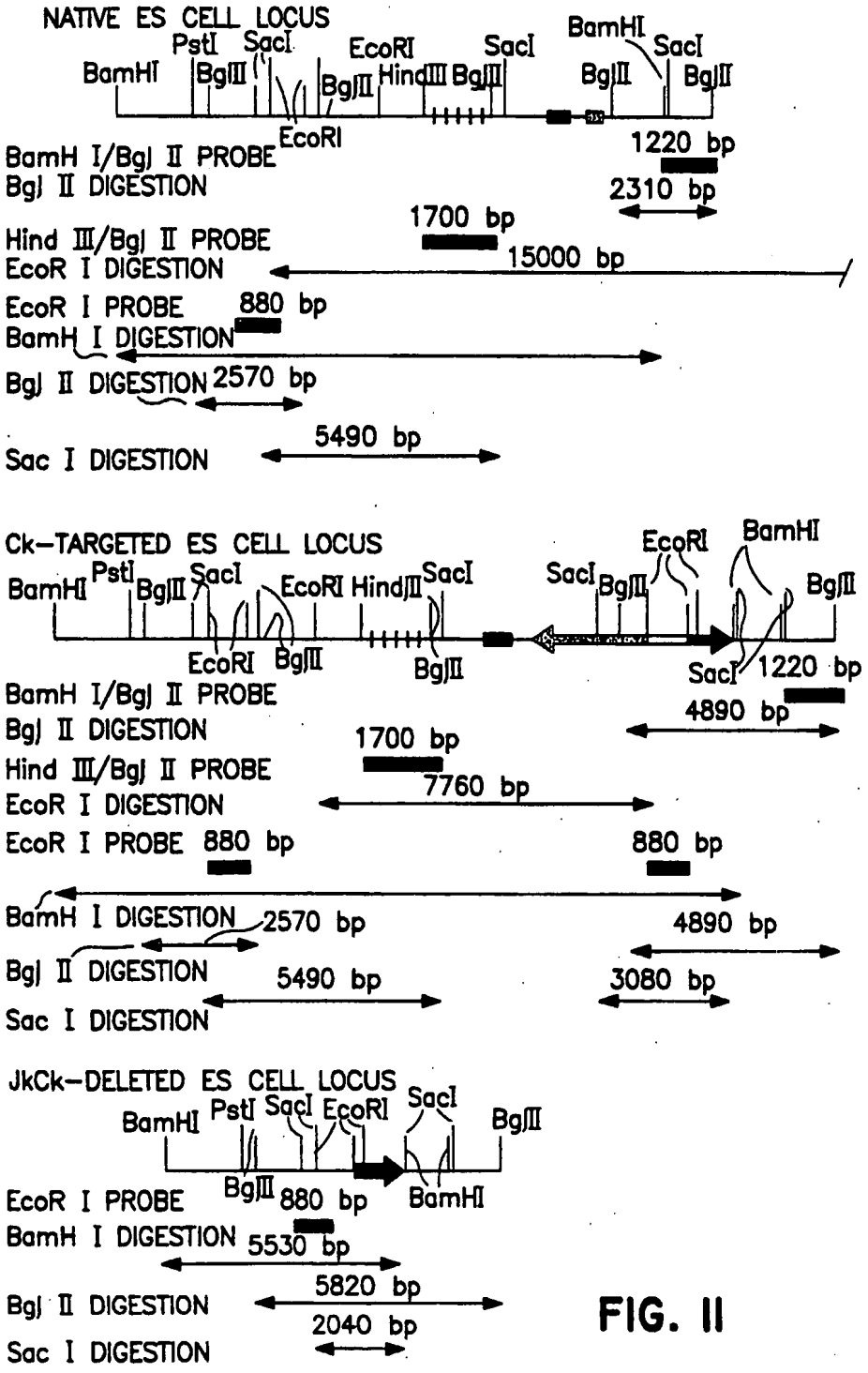
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BamH I/Bgl II PROBE
Bgl II DIGESTION
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FIG. II

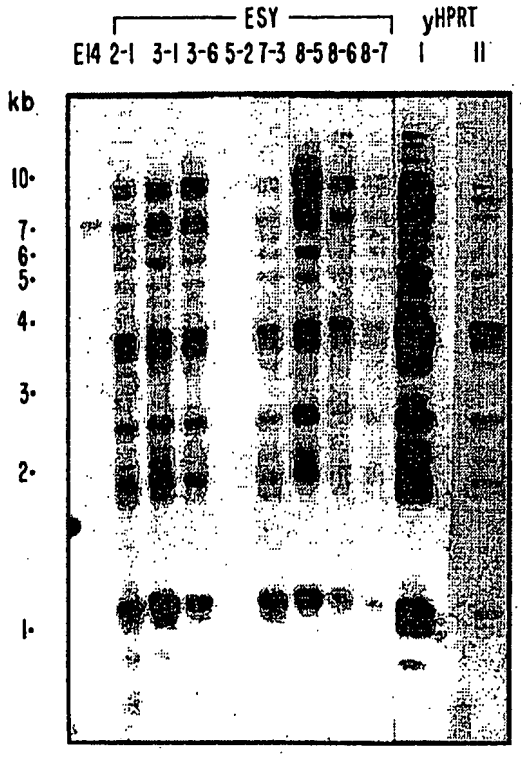


FIG. 12A

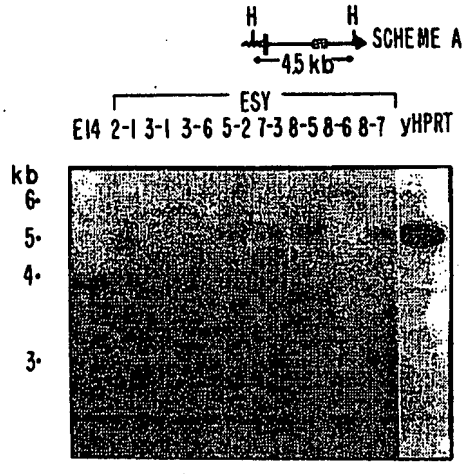


FIG. 12B

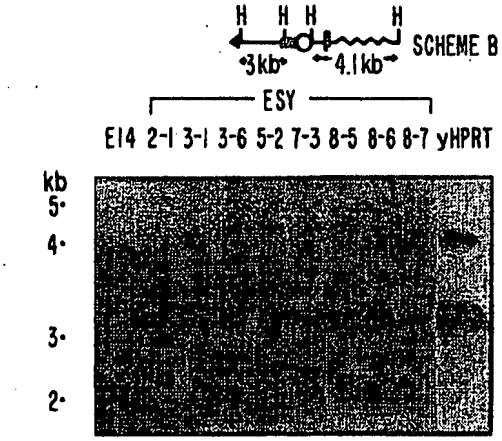


FIG. 12C

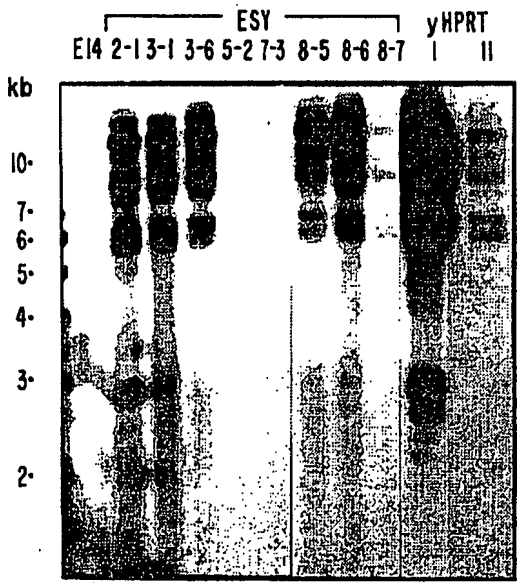


FIG. 12D

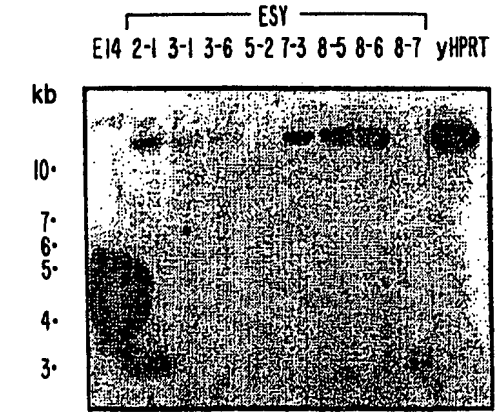


FIG. 12E

FIG. 13A

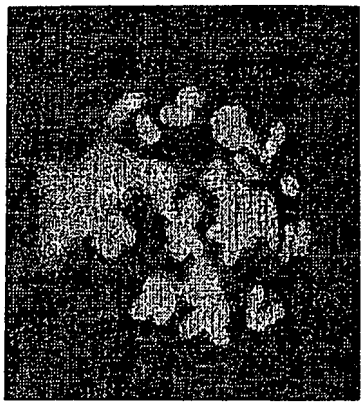
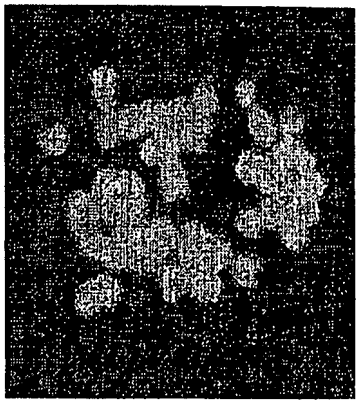


FIG. 13B



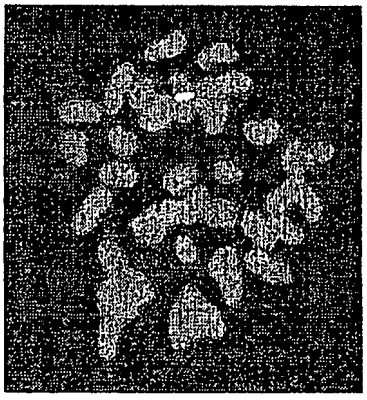


FIG. 13C

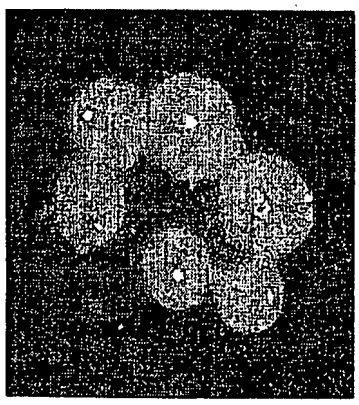


FIG. 13D

FIG. 14A

FIG. 14B

FIG. 14C

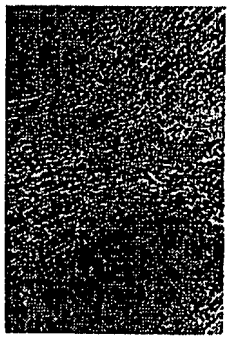


FIG. 14D

FIG. 14E

FIG. 14F

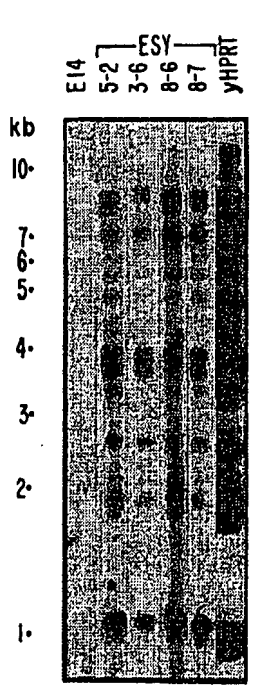


FIG. 14G

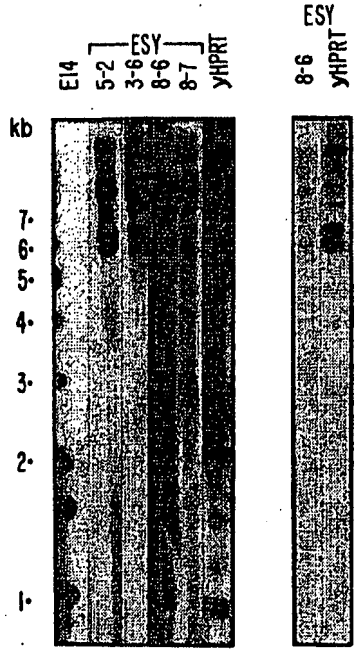


FIG. 14H FIG. 14I

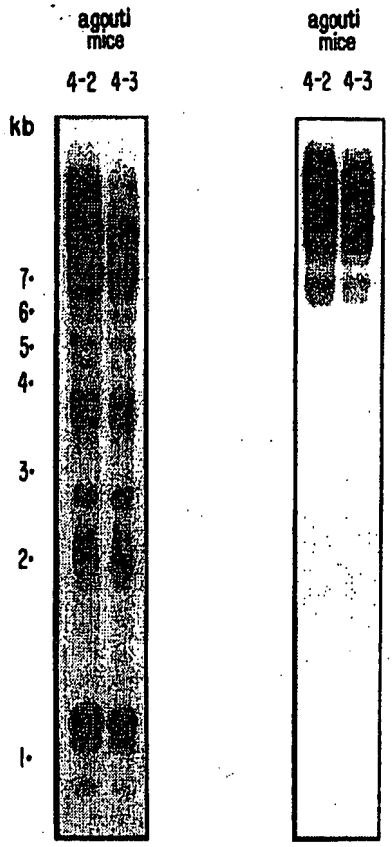


FIG. 14J

FIG. 14K

FIG. 15A

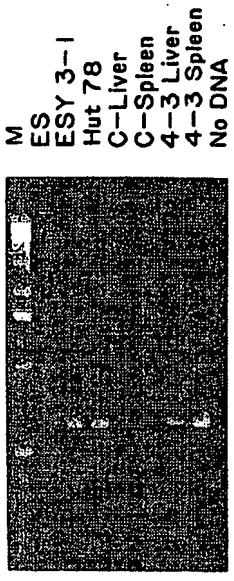
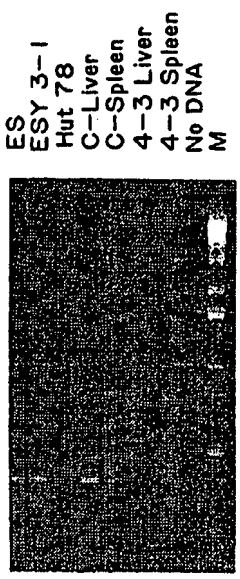


FIG. 15B



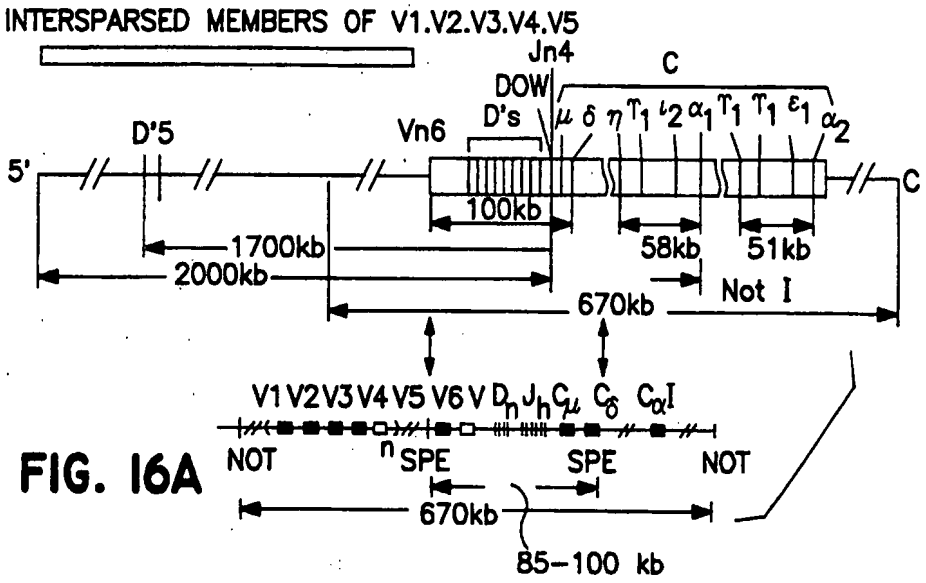


FIG. 16A

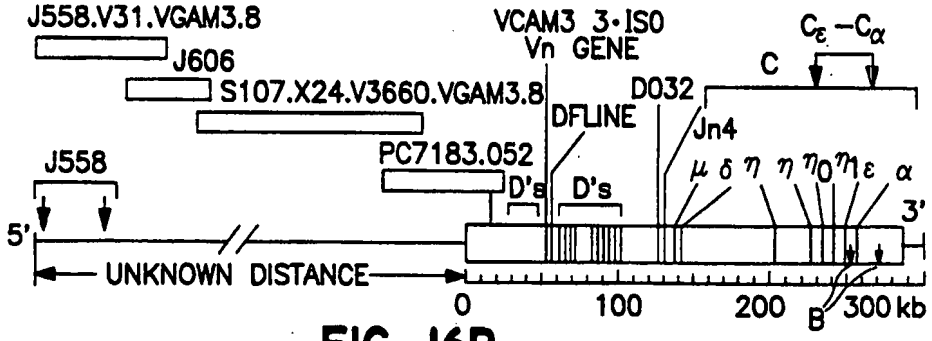


FIG. 16B

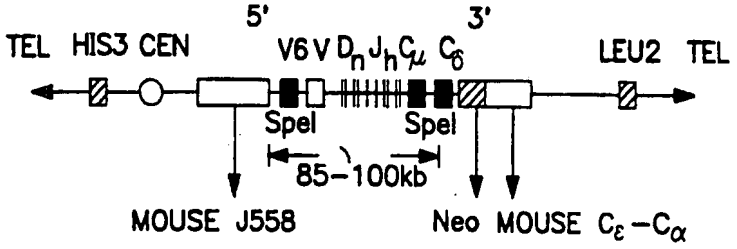


FIG. 16C

Mouse Breeding Scheme

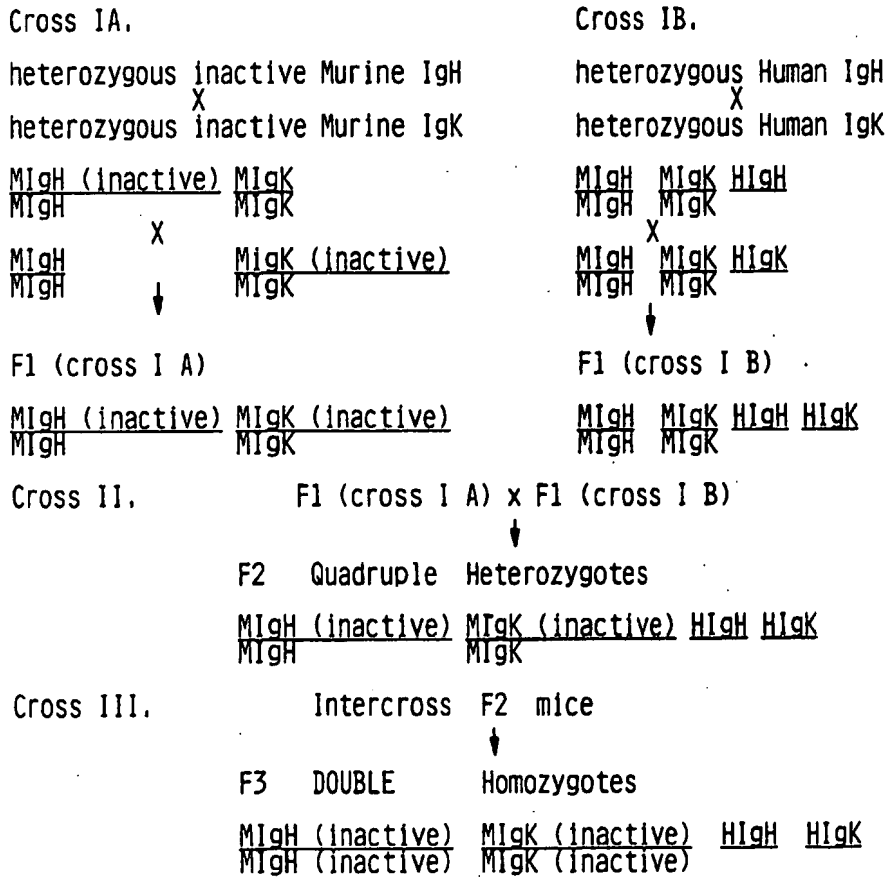


FIG. 17

MAMMALIAN HOST GENOTYPES

FIG. 18A

	Hetero- or Hemi-zygous Mice	Intercross Product Mice *
I.	$\frac{\Delta mIgL}{mIgL}\ \frac{mIgH}{mIgH}$	$\frac{\Delta mIgL}{\Delta mIgL}\ \frac{mIgH}{mIgH}$
II.	$\frac{mIgL}{mIgL}\ \frac{\Delta mIgH}{mIgH}$	$\frac{mIgL}{mIgL}\ \frac{\Delta mIgH}{\Delta mIgH}$
III.	$\frac{mIgL}{mIgL}\ \frac{mIgH}{mIgH}\ \underline{hIgH}$	$\frac{mIgL}{mIgL}\ \frac{mIgH}{mIgH}\ \frac{hIgH}{hIgH}$
IV.	$\frac{mIgL}{mIgL}\ \frac{mIgH}{mIgH}\ \underline{hIgL}$	$\frac{mIgL}{mIgL}\ \frac{mIgH}{mIgH}\ \frac{hIgL}{hIgL}$
V.	Animal I X Animal II	
	$\frac{\Delta mIgL}{mIgL}\ \frac{mIgH}{\Delta mIgH}$	$\frac{\Delta mIgL}{\Delta mIgL}\ \frac{\Delta mIgH}{\Delta mIgH}$
VI.	Animal III X Animal V	
	$\frac{mIgL}{\Delta mIgL}\ \frac{mIgH}{\Delta mIgH}\ \underline{hIgH}$	$\frac{\Delta mIgL}{\Delta mIgL}\ \frac{\Delta mIgH}{\Delta mIgH}\ \frac{hIgH}{hIgH}$ and $\frac{\Delta mIgL}{\Delta mIgL}\ \frac{\Delta mIgH}{\Delta mIgH}\ \underline{hIgH}$
VII.	Animal IV X Animal V	
	$\frac{mIgL}{\Delta mIgL}\ \frac{mIgH}{\Delta mIgH}\ \underline{hIgL}$	$\frac{\Delta mIgL}{\Delta mIgL}\ \frac{\Delta mIgH}{\Delta mIgH}\ \frac{hIgL}{hIgL}$ and $\frac{\Delta mIgL}{\Delta mIgL}\ \frac{\Delta mIgH}{\Delta mIgH}\ \underline{hIgL}$
VIII.	Animal VI X Animal VII	
	$\frac{\Delta mIgL}{\Delta mIgL}\ \frac{\Delta mIgH}{\Delta mIgH}\ \underline{hIgL}\ \underline{hIgH}$	$\frac{\Delta mIgL}{\Delta mIgL}\ \frac{\Delta mIgH}{\Delta mIgH}\ \frac{hIgL}{hIgL}\ \frac{hIgH}{hIgH}$
	$\frac{mIgL}{\Delta mIgL}\ \frac{mIgH}{\Delta mIgH}\ \underline{hIgL}\ \underline{hIgH}$	$\frac{\Delta mIgL}{\Delta mIgL}\ \frac{\Delta mIgH}{\Delta mIgH}\ \frac{hIgL}{hIgL}\ \frac{hIgH}{hIgH}$ and $\frac{\Delta mIgL}{\Delta mIgL}\ \frac{\Delta mIgH}{\Delta mIgH}\ \underline{hIgL}\ \underline{hIgH}$

IX. Animal III X Animal IV

$$\frac{m\mathrm{IgL}}{m\mathrm{IgL}} \quad \frac{m\mathrm{IgH}}{m\mathrm{IgH}} \quad \frac{h\mathrm{IgL}}{h\mathrm{IgL}} \quad \frac{h\mathrm{IgH}}{h\mathrm{IgH}}$$

X. Animal II X Animal IX

$$\frac{m\mathrm{IgL}}{m\mathrm{IgL}} \quad \frac{\Delta m\mathrm{IgH}}{\Delta m\mathrm{IgH}} \quad \frac{h\mathrm{IgL}}{h\mathrm{IgL}} \quad \frac{h\mathrm{IgH}}{h\mathrm{IgH}} \text{ and } \frac{m\mathrm{IgL}}{m\mathrm{IgL}} \quad \frac{\Delta m\mathrm{IgH}}{\Delta m\mathrm{IgH}} \quad \frac{h\mathrm{IgL}}{h\mathrm{IgL}} \quad \frac{h\mathrm{IgH}}{h\mathrm{IgH}}$$

XI. Animal I X Animal IX

$$\frac{\Delta m\mathrm{IgL}}{\Delta m\mathrm{IgL}} \quad \frac{m\mathrm{IgH}}{m\mathrm{IgH}} \quad \frac{h\mathrm{IgL}}{h\mathrm{IgL}} \quad \frac{h\mathrm{IgH}}{h\mathrm{IgH}} \text{ and } \frac{\Delta m\mathrm{IgL}}{\Delta m\mathrm{IgL}} \quad \frac{m\mathrm{IgH}}{m\mathrm{IgH}} \quad \frac{h\mathrm{IgL}}{h\mathrm{IgL}} \quad \frac{h\mathrm{IgH}}{h\mathrm{IgH}}$$

*Not all possible genotypes from intercrosses are shown.

Δ = functionally inactive locus
m = mouse endogenous gene
h = human transgene
IgH = immunoglobulin heavy chain
IgL = immunoglobulin light chain

FIG. 18B

INTERNATIONAL SEARCH REPORT

Int. ational application No.
PCT/US93/06926

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :C12N 15/00
US CL :800/2

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 800/2

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, Dialog, "homologous recombination", ES cells, YACS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Proceedings of the National Academy of Sciences, volume 86, issued August 1989, Traver et al., "Rapid screening of a human genomic library in yeast artificial chromosomes for single-copy sequences", pages 5898-5902, see entire article.	1-11
Y	Proceedings of the National Academy of Sciences, volume 87, issued July 1990, Pachnis et al., "Transfer of a yeast articial chromosome carrying human DNA from Saocharomyoes cerevisiae into mammalian cells", pages 5109-5113, see entire article.	1-11

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be part of particular relevance

"E" earlier document published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
13 SEPTEMBER 1993	21 OCT 1993
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	Authorized officer SUZANNE ZISKA
Facsimile No. NOT APPLICABLE	Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

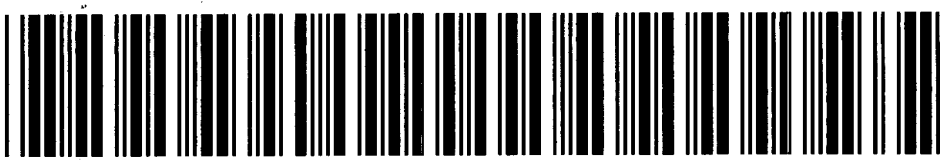
INTERNATIONAL SEARCH REPORT

Int...national application No.
PCT/US93/06926

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Nature, volume 338, issued 09 March 1989, Joyner et al., "Production of a mutation in mouse En-2 gene by homologous recombination in embryonic stem cells", pages 153-156, see entire article.	1-11

IDS REFERENCES



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Dimerization of Cell Surface Receptors in Signal Transduction

Review

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Biomedical Center
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Sweden

Introduction

Cell growth, differentiation, migration, and apoptosis are in part regulated by polypeptide growth factors or cytokines. As these factors are unable to pass the hydrophobic cell membrane, a fundamental question is how they transduce their signals into the cell. Growth factors and cytokines exert their effects via binding to cell surface receptors; results obtained during recent years have given ample evidence that such receptors often are activated by ligand-induced dimerization or oligomerization. Moreover, the elucidation of intracellular signal transduction pathways have revealed that the activity of several components in these pathways are also regulated by dimerization. For instance, certain of the cytoplasmic signal transduction molecules dimerize after activation, and the active form of transcription factors are often dimers. It thus appears that dimerization is a mechanism of general applicability for the regulation of signal transduction.

This review focuses on the role of dimerization of cell surface receptors in signal transduction. Dimerization or oligomerization have been shown to occur after binding of several polypeptide hormones, cytokines, growth factors, or growth inhibitors to their receptors. Examples include protein-tyrosine kinase receptors, cytokine receptors, antigen receptors, receptors for tumor necrosis factor (TNF) and related factors, and serine/threonine kinase receptors (Figure 1; Table 1). There are, however, many variations on the theme, as will be discussed below.

Protein-Tyrosine Kinase Receptors

Many traditional growth factors, such as platelet-derived growth factor (PDGF), epidermal growth factor (EGF), and fibroblast growth factor (FGF), bind to receptors with tyrosine kinase activity (Table 1). Protein-tyrosine kinase receptors consist of single transmembrane domains separating the intracellular kinase domains from extracellular domains, which typically contain one or several copies of immunoglobulin-like domains, fibronectin type III–like domains, EGF-like domains, cysteine-rich domains, or other domains (reviewed by Fanti et al., 1993). Based on their structural characteristics, the tyrosine kinase receptors can be classified into families; the largest families are listed in Table 1.

Several of the ligands for protein-tyrosine kinase receptors are dimeric molecules, which thus contain two identical receptor-binding epitopes. Examples include PDGF and colony-stimulating factor 1 (CSF-1), which are disulfide-bonded dimers, and stem cell factor (SCF), which is a dimer held together by noncovalent forces. These ligands form stable receptor dimers by simultaneously binding two receptors. In addition to the bridging of the ligand between two receptors, it is possible that direct interactions between the receptors, involving epitopes located outside the ligand-binding domains, are important for stabilization of the receptor dimer. In the case of the SCF receptor, evidence has been presented that epitopes in the fourth immunoglobulin domain are involved in such receptor–receptor interactions (Blechman et al., 1995). It is possible that such direct receptor–receptor interactions are promoted by conformational changes in the receptors induced by ligand binding. Other ligands, like EGF, have apparent monomeric configurations; interestingly, however, recent calorimetric studies have shown that a single EGF molecule also can bind simultaneously to two receptor molecules (Lemmon and Schlessinger, 1994). Another variation on the theme is exemplified by ligands for Eph-related tyrosine kinase receptors. These ligands are cell surface attached and do not activate receptors in soluble form. The possibility that receptor dimerization or clustering is involved in receptor activation, presumably facilitated by membrane attachment of ligands, is supported by the finding that antibody-mediated clustering of soluble receptors led to activation of receptors (Davis et al., 1994).

Receptor Autophosphorylation

Dimerization of protein-tyrosine kinase receptors is followed by receptor "autophosphorylation," which mainly occurs by one receptor molecule phosphorylating the other in the dimer (Ullrich and Schlessinger, 1990). The autophosphorylation occurs on two principally different classes of tyrosine residues. On one hand, autophosphorylation is commonly seen on a conserved tyrosine residue within the kinase domains (Tyr-857 in the PDGF β receptor; Figure 2). In the cases of the receptors for insulin and hepatocyte growth factor (HGF), phosphorylation of the tyrosine residue at this and neighboring sites leads to an increase in the kinase activity and precedes phosphorylation of other sites in the receptor or substrates (Naldini et al., 1991; White et al., 1988). This thus appears to be an allosteric site that regulates the V_{max} of the receptor kinase. It is still not known how the autophosphorylation is initiated; one possibility is that the monomeric receptor has a low basal kinase activity, which is sufficient to phosphorylate and activate the companion receptor after dimerization. This would then rapidly be followed by reciprocal phosphorylation. Alternatively, the interaction between the intracellular domains of the receptors in the dimer may induce a conformational change that leads to an increased kinase activity. Not all receptors are regulated by phosphorylation inside the kinase domain, e.g., in the EGF receptor, the conserved tyrosine residue in the kinase domain appears not to be autophosphorylated.

The other class of autophosphorylation sites are normally localized outside the kinase domains and serve the important function of creating docking sites for downstream signal transduction molecules containing Src-homology 2 (SH2) domains. The SH2 domains consists of about 100 amino acid residues folded in such a way

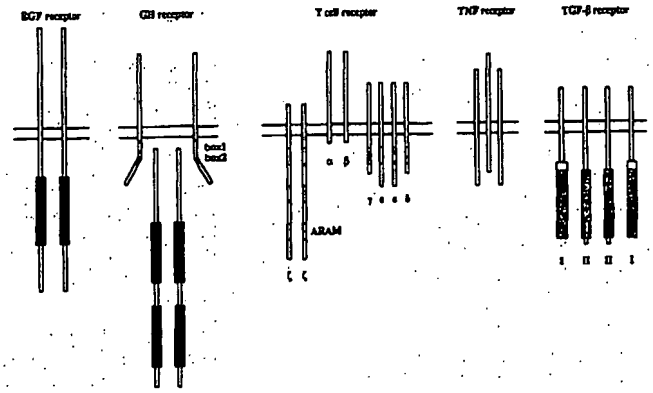

Figure 1. Examples of Receptors Activated by Dimerization or Oligomerization

Schematic representations of the complexes formed after ligand binding to receptors from the families discussed in the text, i.e., protein-tyrosine kinase receptors (the EGF receptor shown as an example), cytokine receptors (GH receptor bound to JAK kinases), antigen receptors (TCR), trimeric receptors (TNF receptor), and serine/threonine kinase receptors (TGFβ receptor). Tyrosine kinase domains are closed and serine/threonine kinase domains dark stippled. Box1, box2 (light stippled), refers to a region in cytokine receptors to which JAK kinases bind. ARAM (light stippled) refers to antigen recognition activation motifs in different subunits of antigen receptors that become phosphorylated by tyrosine kinases of the Src family and thereafter bind tyrosine kinases of the ZAP-70/Syk family.

Table 1. Families of Receptors Activated by Dimerization or Oligomerization

Receptor Type	Family	Examples	Characteristics
Protein-tyrosine kinase receptors	PDGF receptor family	PDGFR-α, PDGFR-β, SCFR (Kit), CSF-R (Fms), Flk-2	Five immunoglobulin-like domains extracellularly
	EGF receptor family	EGFR (ErbB), ErbB2 (Neu), ErbB3, ErbB4	Two cysteine-rich domains extracellularly
	FGF receptor family	FGFR-1, FGFR-2, FGFR-3, FGFR-4	Two to three immunoglobulin-like domains extracellularly
	IGF receptor family	Insulin R, IGF-1R	Disulphide-bound heterotetramer of α and β chains
	HGF receptor family	HGFR (Met), MSPR (Ron)	Extracellular domain cleaved into an α and β chain
	VEGF receptor family	Flt-1, Flk-1 (KDR)	Seven immunoglobulin-like domains extracellularly
	Neurotrophin receptor family	Trk, TrkB, TrkC	
	Eph receptor family	Eph, Elk, Eck, Cck5, Sek, Eck, Erk	Two FNIII-like domains and a cysteine-rich domain extracellularly
Cytokine receptors	Class I cytokine receptor family		
	GH receptor subfamily	GHR, EPOR, PRLR, G-CSFR	Form homodimers
	IL-3 receptor subfamily	IL-3R, GM-CSFR, IL-5R	Form complexes with the β_c subunit
	IL-6 receptor subfamily	IL-6R, LIFR, CNTFR, IL-11R	Form complexes with gp130
	IL-2 receptor subfamily	IL-2Rα, IL-2rβ, IL-4R, IL-7R	Form complexes with IL-2Rγ
	Class II cytokine receptor family	IFN-α/βR, IFN-γRα, IFN-γRβ, IL-10R	
TNF receptor family		TNFR-1, TNFR-II, LNGFR, CD40, OX-40, Fas, CD27, CD30	Form trimers
Antigen receptors		TCR	Complex of α, β, γ, δ, ε, ζ an η subunits
		BCR	Complex of IgM and heterodimers of α/β subunits
Serine/threonine kinase receptor family	Type II receptor family	TGFβR-II, ActR-II, ActR-IIB	Form hetero-oligomers with type I receptors, i.e., TGFβR-I, ActR-1, ActR-1B, BMPR-IA, BMPR-IB, ALK-1

Receptor families and subfamilies discussed in the text are presented. Abbreviations used: R, receptor; PDGF, platelet-derived growth factor; SCF, stem cell factor; CSF, colony-stimulating factor; EGF, epidermal growth factor; FGF, fibroblast growth factor; IGF, insulin-like growth factor; HGF, hepatocyte growth factor; MSP, macrophage-stimulating protein; VEGF, vascular endothelial growth factor; FN, fibronectin; GH, growth hormone; EPO, erythropoietin; PRL, prolactin; IL, interleukin; LIF, leukemia inhibitory factor; CNTF, ciliary neurotrophic factor; IFN, interferon; TNF, tumor necrosis factor; LNGFR, low affinity nerve growth factor receptor; TCR, T cell receptor; BCR, B cell receptor; TGFβ, transforming growth factor β; Act, activin; BMP, bone morphogenic protein. Alternative designations are given within parentheses.

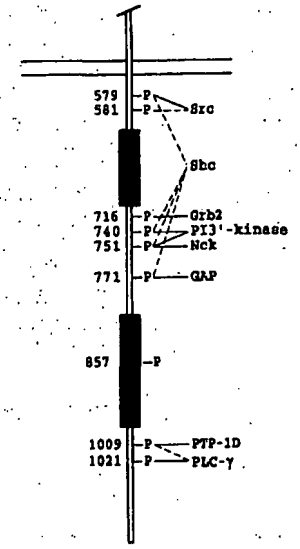

Figure 2. Interaction of SH2 Domain–Containing Signal Transduction Molecules with Different Autophosphorylation Sites in the PDGF β Receptor

Schematic illustration of the intracellular portion of a PDGF β receptor after activation. The kinase domain (closed boxes) in the receptor is divided into two parts by an inserted sequence. The tyrosine residues in the receptor known to be autophosphorylated are indicated by numbers. The interaction of individual autophosphorylated tyrosine residues with different SH2 domain–containing proteins are also indicated. Shc, Grb2, and Nck are adaptor molecules; Src denotes different members of the Src family of tyrosine kinases; PI3-kinase, phosphatidylinositol 3′-kinase; GAP, GTPase-activating protein; PTP1D, protein tyrosine phosphatase 1D; PLC-γ, phospholipase C-γ.

that a binding pocket for a phosphorylated tyrosine and the immediately surrounding amino acid residues is formed (Pawson and Schlessinger, 1993; Cohen et al., 1995 [this issue of *Cell*]). Of particular importance are the three to six amino acid residues C-terminal of the phosphorylated tyrosine (Eck et al., 1993; Pascal et al., 1994; Waksman et al., 1993); since different SH2 domains have different preferences for this region, there is specificity in the interaction. As an example, the PDGF β receptor has been shown to contain at least nine autophosphorylated tyrosine residues; Tyr-857 in the second part of the kinase domain is of importance for the catalytic activity of the kinase, whereas the others interact in a specific manner with at least eight different signal transduction molecules (reviewed by Claesson-Welsh, 1994; Figure 2).

Homodimerization or Heterodimerization

Protein-tyrosine kinase receptors are activated after homodimerization or after heterodimerization. In the case of the PDGF receptor subfamily, the different isoforms of PDGF induce different dimeric forms of the receptors. Since the A chain of PDGF binds only α receptors while the B chain binds both α and β receptors with high affinity, PDGF-AA induces αα receptor homodimers only, PDGF-AB induces αα receptor homodimers and αβ receptor heterodimers, and PDGF-BB induces all three combinations of receptors (Heldin et al., 1989; Kanakaraj et al., 1991; Seifert et al., 1989). There are certain differences in the signals transduced via αα receptor homodimers and ββ receptor homodimers, e.g., regarding the stimulation of chemotaxis and actin reorganization. Moreover, PDGF-AB, which preferentially induces αβ receptor dimers, induces a stronger mitogenic response than the other PDGF isoforms. A possible explanation for the unique properties of the αβ receptor heterodimer is the presence of unique autophosphorylation sites, not seen in the homodimeric receptors, and that may mediate interactions with additional signal transduction molecules (Rupp et al., 1994). Thus, the response to PDGF depends both on the particular isoform of PDGF and on the number of α and β receptors expressed on the target cells.

The EGF receptor was the first protein-tyrosine kinase receptor to be shown to dimerize after ligand binding (Yarden and Schlessinger, 1987). However, within the same subfamily of tyrosine kinase receptors, heterodimerization of receptors has also been observed. A candidate ligand for ErbB2 (Neu differentiation factor [NDF], also called heregulin, glial growth factor, or acetylcholin-receptor-inducing activity), which is structurally related to EGF, was found to induce heterodimeric complexes between ErbB2 and ErbB3 or ErbB4 (Peles et al., 1993; Plowman et al., 1993; Sliwkowski et al., 1994). Moreover, the presence of ErbB3 or ErbB4 was necessary for high affinity binding of NDF and signal transduction through ErbB2 to occur. Interestingly, ErbB3 lacks certain highly conserved amino acid residues in its kinase domain; consistent with this finding, ErbB3 was found to have low or no kinase activity (Prigent and Gullick, 1994). It is thus possible that the major function of ErbB3 in the heterodimer is to act as a substrate for the ErbB2 kinase and thus provide docking sites for downstream SH2 domain–containing signal transduction molecules (Carraway and Cantley, 1994); for example, binding motifs for the SH2 domains of the phosphatidylinositol 3′-kinase (PI3-kinase) are lacking in the EGF receptor and in ErbB2, but occur in several copies in ErbB3 (Fedi et al., 1994; Soltoff et al., 1994). Also, EGF itself can induce heterodimerization of EGF receptors and ErbB2 (Soltoff et al., 1994; Wada et al., 1990). In fact, heterodimerization is preferred in cells expressing both EGF receptors and ErbB2. Although heterodimerization occurred also with a kinase-inactivated ErbB2 receptor mutant, this complex was inactive, showing that in this case signaling can not occur via ErbB2 serving as a EGF receptor substrate (Qian et al., 1994).

The studies on dimerization of receptors in the PDGF receptor and EGF receptor families thus provide examples of different types of dimeric complexes induced after ligand binding, i.e., homodimeric (Figure 3A) or heterodimeric (Figure 3B) complexes between two catalytically active subunits, or a heterodimeric complex between one active and one inactive or less active subunit (Figure 3C). Given that tyrosine kinase receptors and ligands occur in families of structurally related molecules, it is not unlikely that homodimerization and heterodimerization of receptors occur in parallel also in other families, thus increasing

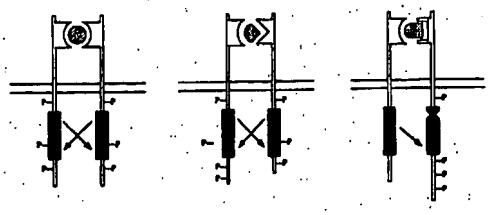

Figure 3. Different Dimeric Complexes of Protein-Tyrosine Kinase Receptors

Schematic representation of different forms of dimeric complexes of tyrosine kinase receptors formed after ligand binding. (A) a homodimeric complex; (B) a heterodimeric complex of two kinase-active subunits; (C) a heterodimeric complex of one active and one inactive or less active subunit.

the range of responses possible from a given number of receptor molecules.

One special case is the insulin and insulin-like growth factor 1 (IGF-1) receptor family. These receptors exist in the cell membrane as disulfide-bonded homo- or heterodimers of receptor subunits (each subunit is further cleaved into α and β chains by proteolysis) (Soos and Siddle, 1989). Thus, ligand binding does not induce receptor dimerization, but presumably causes a conformational alteration in the preformed dimeric receptor, which leads to receptor activation. Moreover, autophosphorylated tyrosine residues in the receptor molecules are not so important for the binding of downstream components in the signal transduction pathways; rather, the insulin receptor kinase phosphorylates insulin receptor substrate 1 (IRS-1), which mediates the interactions with SH2 domain proteins (White, 1994).

Cytokine Receptors

The cytokine receptor classes include receptors for many interleukins, colony-stimulating factors, interferons, and certain other factors and hormones (reviewed by Kishimoto et al., 1994; Mui and Miyajima, 1994; see Table 1). Class I cytokine receptors are characterized by the presence in their extracellular domains of one or two copies of a conserved domain of about 200 amino acids, which contains two modules of fibronectin type III–like motifs, four conserved cysteine residues, and the conserved motif Trp–Ser–Xaa–Trp–Ser (Bazan, 1990). Class II cysteine receptors, including receptors for interferons and interleukin-10 (IL-10), contain another conserved motif of four cysteine residues and lack the Trp–Ser–Xaa–Trp–Ser motif. The intracellular domains of cytokine receptors lack intrinsic enzymatic activities. However, despite the structural difference between cytokine receptors and tyrosine kinase receptors, their mechanism of activation appears to be similar. Ligand binding induces dimerization or oligomerization of cytokine receptors, and this allows interaction and activation of cytoplasmic protein-tyrosine kinases that are associated with the intracellular domain of the receptors.

Activation of Class I Cytokine Receptors through Formation of Hetero-Oligomeric Complexes

Most of the class I cytokine receptors undergo heterodimerization or hetero-oligomerization after ligand binding (Table 1). In many cases, the ligand-binding subunit(s) form signaling complexes with signal-transducing molecules that are structurally related to cytokine receptors, but that are themselves unable to bind ligands. For instance IL-3, granulocyte/macrophage colony-stimulating factor (GM-CSF), and IL-5 bind to specific α subunit receptors; the α subunits all interact with a common β subunit that is required for high affinity ligand binding and signal transduction (Mui and Miyajima, 1994).

Similarly, IL-6, leukemia inhibitory factor (LIF), oncostatin M, IL-11, and ciliary neurotrophic factor (CNTF) share a common signal transducer, gp130 (Taga et al., 1989); signaling is triggered by the formation of homo- or heterodimers of gp130. IL-6 binds to the IL-6 receptor and induces a complex containing a homodimer of gp130 (Murakami et al., 1993). Interestingly, signaling occurs also with a truncated IL-6 receptor lacking the cytoplasmic domain, which indicates that the IL-6 receptor is needed only to increase the binding affinty for IL-6. The CNTF receptor acts similarly, lacking a cytoplasmic domain in its natural form and being anchored in the membrane through a phosphatidylinositol group. The CNTF receptor–CNTF complex signals via formation of a heteromeric complex of gp130 and the LIF receptor (Davis et al., 1993). LIF and oncostatin M signal via binding directly to a heteromeric complex of gp130 and the LIF receptor (Gearing et al., 1992). IL-11 is dependent on gp130 but not the LIF receptor for signaling (Hilton et al., 1994).

A third subfamily is constituted by IL-2, IL-4, IL-7, and IL-9. In this family, signaling involves the formation of heterodimeric receptor complexes between specific β subunits and a common γ subunit (Kawahara et al., 1994). In the case of IL-2, the ligand binding affinity is increased by the presence also of an α subunit, which has a structure unrelated to that of cytokine receptors. Whereas the α subunit is not needed for signal transduction, both the β and the γ subunits are needed, presumably in a heterodimeric configuration (Nakamura et al., 1994; Nelson et al., 1994).

Activation by Homodimerization

Although activation by heterodimerization appears to be most common among cytokine receptors, there are examples of cytokine receptors that are activated by homodimerization, e.g., the receptors for growth hormone (GH), erythropoietin (EPO), prolactin, and granulocyte colony-stimulating factor (G-CSF) (Table 1). A well-characterized example is the GH receptor. Analysis of crystals of GH and the extracellular part of the receptor revealed that each ligand binds two receptor molecules simultaneously (Cunningham et al., 1991; de Vos et al., 1992; Ultsch et al., 1991). This finding was surprising since GH is a monomeric molecule without apparent symmetry. The two receptor-binding sites in GH are therefore different, although they bind to similar epitopes in the receptors. Site 1 is larger and is supposed to bind receptor first; the smaller site 2 thereafter binds a second receptor, and the dimeric receptor complex is further stabilized by direct interaction between the two receptors. The importance of the latter

epitope in stabilizing a GH receptor dimer is illustrated by the finding that a mutation in this region abolishes receptor homodimerization and is responsible for a form of familial GH resistance (Laron's syndrome; Duquesnoy et al., 1994). The results from the three-dimensional structure studies are supported by titration calorimetry in solution; the heat of binding was found to be saturated at a 1:2 ratio of ligand and receptor (Ultsch et al., 1991).

Signal Transduction

Much information regarding the signal transduction pathways from cytokine receptors to the nucleus has come from a genetic approach in which mutant cell lines defective in the response to interferons were isolated and characterized (Darnell et al., 1994). This approach led to the identification of three categories of proteins, a DNA-binding protein (p48), STATs (signal transducers and activators of transcription), and cytoplasmic protein-tyrosine kinases of the JAK family.

The JAK kinases are characterized by the presence of two kinase domains in each molecule, which is the basis for their name (Janus kinases, after the Roman god with two faces) (reviewed by Ziemiecki et al., 1994). Several members of this family are currently known (JAK1, JAK2, Tyk2, JAK3 [Ziemiecki et al., 1994; Takahashi and Shirasawa, 1994]); they associate in a specific manner with different cytokine receptors and are activated upon receptor dimerization. An important class of substrates for JAK kinases is members of the STAT family (STAT1α, STAT1β, STAT2, STAT3, STAT4, STAT5, and IL-4 STAT) (Darnell et al., 1994; Zhong et al., 1994; Gouilleux et al., 1994; Hou et al., 1994). After phosphorylation on tyrosine residues, the STAT molecules form homo- or heterodimers.

In the case of signaling from the interferon-α receptor, heterodimers of STAT1α (p91) or STAT1β (p84) and STAT2 (p113) are created, which move into the nucleus and form a complex with a DNA-binding protein (p48), allowing them to bind and stimulate transcription from elements in the promoters of interferon-α-induced genes (Schindler et al., 1992). Interestingly, another combination of STATs is formed after stimulation by interferon-γ, either a homodimer of STAT1α (p91) or a homodimer of STAT1β (p84) (Shuai et al., 1994; Shuai et al., 1992). These dimers do not associate with p48, but may form complexes with other related proteins. Both homodimers bind to interferon-γ-activated sites (GAS), which are present in interferon-γ-inducible genes, although only the STAT1α homodimer activates transcription (Shuai et al., 1993).

It thus appears that the activities of STATs are regulated by specific assembly into homo- or heterodimers. The dimerization is triggered by phosphorylation. A single phosphorylated tyrosine residue has been identified in STAT1 after stimulation with interferon-α as well as after stimulation with interferon-γ; mutation of this tyrosine residue to a phenylalanine residue prevents dimerization (Shuai et al., 1993). Since STATs contain SH2 domains, it is likely that the dimerization involves reciprocal interactions between the SH2 domains and the tyrosine-phosphorylated regions in the STAT molecules (Shuai et al., 1994). How is the specificity regulated? One possibility is that STATs may associate in a differential manner with different receptors (Fu and Zhang, 1993; Greenlund et al., 1994).

Another possibility is that the JAK family members differ in their substrate specificities and thus phosphorylate different STAT molecules. Interestingly, the genetic approach led to the identification of different JAK kinases in the signaling pathways of interferon-α (JAK1 and Tyk2) and interferon-γ (JAK1 and JAK2) (Müller et al., 1993; Velazques et al., 1992; Watling et al., 1993). Thus, in each case, there was a need for two different JAK kinases. It is unlikely that the two kinases are needed in a sequential activation mechanism, since in cells deficient in JAK1 no activation of JAK2 was seen after stimulation with interferon-γ, or vice versa (Müller et al., 1993). Thus, it is possible that the active forms of the JAK kinases involved in the signal pathways of interferons are activated by heteromeric interactions, possibly involving cross-phosphorylations.

A possible mechanism to achieve such heterodimerization is via ligand-dependent formation of heteromeric receptor complexes. The receptor for interferon-γ consists of at least two different chains (Aguet et al., 1988; Hemmi et al., 1994; Soh et al., 1994), and it has been suggested that JAK1 and JAK2 interact with these chains in a differential manner (Greenlund et al., 1994). An interferon-α/β receptor that binds JAK1 has been identified (Novick et al., 1994); whether another receptor subunit with affinity for Tyk2 exists remains to be elucidated. An analogous situation appears to prevail for the IL-2 receptors; the β and γ subunits have been shown to bind JAK1 and JAK3, respectively (Miyazaki et al., 1994; Russell et al., 1994).

Common and Unique Signals

In addition to the receptors for interferon-α and interferon-γ, many other receptors, including GH, EPO, prolactin, G-CSF, LIF, gp130, the common β subunit for the IL-3 subfamily of receptors, and the common γ subunit for the IL-2 subfamily, have been shown to bind different members of the JAK family (Ihle et al., 1994). JAKs bind in a specific manner to conserved regions called box 1 and box 2 regions in the juxtamembrane parts of cytokine receptors (Murakami et al., 1991).

Other signal transduction pathways are also initiated at the activated cytokine receptor complexes; these pathways are dependent on more C-terminal regions in the receptors. For instance, members of the Src family of kinases bind to the C-terminal tail of the IL-2 β receptor (Hatakeyama et al., 1991) and to gp130 (Ernst et al., 1994). Moreover, whereas JAK kinases bind to the juxtamembrane part of the common β subunit of IL-3, IL-5, and GM-CSF, deletion of the C-terminus abrogates Shc phosphorylation, Ras activation, and induction of *c-fos* and *c-jun* (Sato et al., 1993). Likewise, a region C-terminal of the JAK kinase–binding site of the G-CSF receptor mediates induction of granulocyte-specific genes (Fukunaga et al., 1993).

The fact that certain receptor subunits/signal transducers are shared by several cytokines, as well as the fact that different receptors may bind and activate the same JAK kinases and possibly also share other signal transduc-

tion molecules, provides an explanation for the functional redundancy and pleiotropy of different cytokines. Conversely, the presence of unique epitopes in receptors or receptor combinations may allow the transduction of specific signals that mediate unique properties of the different cytokines.

Sharing of Signal Transduction Pathways between Tyrosine Kinase Receptors and Cytokine Receptors

There is no sharp division in the modes of signaling between tyrosine kinase receptors and cytokine receptors. EGF and PDGF, acting via tyrosine kinase receptors, induce the phosphorylation of STAT1α, perhaps directly or via activation of JAK kinases (Fu and Zhang, 1993; Ruff-Jamison et al., 1993; Sadowski et al., 1993; Silvennoinen et al., 1993). Moreover, after activation of cytokine receptors, JAK kinases or possibly other kinases phosphorylate the cytokine receptors themselves. This gives SH2 domain–containing signal transduction molecules the possibility to interact with the cytokine receptors and initiate pathways initially identified for tyrosine kinase receptors, e.g., leading to activation of Ras and PI3-kinase (Boulton et al., 1994; Mui and Miyajima, 1994).

Antigen Receptors

The T cell receptor is composed of transmembrane proteins with very short cytoplasmic sequences, which are associated with a large number of invariant subunits also lacking intrinsic enzymatic activities, but capable of interacting with cytoplasmic tyrosine kinases (Figure 1) (for reviews see Cambier and Jensen, 1994; Weiss and Littman, 1994). The invariant subunits (γ, δ, ε, ζ, and η chains) contain one to three copies of a conserved 26 amino acid motif of pairs of tyrosine and leucine residues (Reth, 1989), called the antigen recognition activation motif (ARAM; also called tyrosine-based activation motif, or antigen receptor homology 1). Tyrosine kinases of the Src family bind to the T cell receptor even in the resting state. In conjunction with receptor activation, the tyrosine residues in the ARAMs are phosphorylated, presumably by Src family kinases (in T cells primarily Lck). This gives another tyrosine kinase, ZAP-70, which has two SH2 domains, the opportunity to bind to the phosphorylated sites, after which it becomes activated by phosphorylation on tyrosine residues, most likely also by Src family tyrosine kinases (Iwashima et al., 1994; Letourneur and Klausner, 1992). The precise mechanism that triggers antigen receptor activation and phosphorylation of the ARAM sequences is not known, although a possible scenario is that antigen binding causes receptor aggregation that makes possible interactions and cross-phosphorylation of tyrosine kinases in the Src family. Consistent with this possibility are the observations that chimeric molecules consisting of cytoplasmic parts of ARAM–containing T cell receptor subunits and extracellular domains of other molecules mediate activation of T cells after cross-linking (Letourneur and Klausner, 1992; Irving and Weiss, 1991; Romeo and Seed, 1991).

The B cell receptor and Fc receptors also occur in complexes containing signal transducing molecules with ARAMs, suggesting similar mechanisms of signal transduction (Clark et al., 1994; Law et al., 1993; Ravetch, 1994). Interestingly, a sequential activation of Src family members and ZAP-70/Syk family members may also be involved in cytokine signaling. The G-CSF receptor has been shown to be associated with Lyn, a member of the Src family; after stimulation, an ARAM-like motif in the C-terminus of the G-CSF receptor is phosphorylated, which binds Syk leading to its activation (Corey et al., 1994).

TNF Receptor Family

An interesting variation on the "activation by oligomerization" theme is provided by members of the TNF receptor family, which are involved in regulation of cytotoxicity, apoptosis, and proliferation (for reviews see Bazan, 1993; Smith et al., 1994). TNF occurs as two forms, TNFα and TNFβ, which both binds to two different receptors, TNF receptor 1 and 2 (p55 and p75, respectively). The TNFs are nondisulfide-bonded trimers, and elucidation of the X-ray structure of TNFβ and TNF receptor 1 (Banner et al., 1993) revealed that ligand binding induces trimerization of the receptor. Each TNF subunit makes contact with two adjacent receptor molecules, thus stabilizing the receptor trimer. It is likely that the activating event is receptor aggregation, but it is not clear whether there is a need for receptor trimerization, or whether receptor dimerization would be sufficient for activation. In support of the possibility that trimerization of TNF receptor 1 is, in fact, necessary for signal transduction, monoclonal antibodies against this receptor, which are expected to dimerize the receptor, do not lead to activation, whereas activation occurs after cross-linking of the monoclonals with a second antibody, or after stimulation by two monoclonals directed against different epitopes (Engelmann et al., 1990).

A novel family of molecules that associate with the cytoplasmic part of TNF receptor 2 and that may serve as signal transducers was recently identified (Rothe et al., 1994); TNF receptor associated factors, TRAF1 and TRAF2, contain a novel region of homology and form homo- or heterodimers. This finding represents an important step in the understanding of signaling from the TNF receptor 2, but the mode of activation of TRAFs, their downstream effectors, and whether related molecules are involved in signaling from other members in the TNF receptor family remain to be elucidated.

Protein-Serine/Threonine Kinase Receptors

Transforming growth factor β (TGFβ) is a prototype for a large family of structurally related factors that regulate cell growth and differentiation, including in addition to TGFβs, e.g., activins and inhibins, bone morphogenic proteins, and Müllerian inhibition substance. As far as has been characterized, these molecules exert their cellular effects by binding to heteromeric complexes of serine/threonine kinase receptors (reviewed by Massagué et al., 1994; Miyazono et al., 1994).

Both type I and type II receptors have rather small cysteine-rich extracellular domains; the type I receptors, which are more similar to each other than to the type II receptors, all have a characteristic region rich in glycine and serine

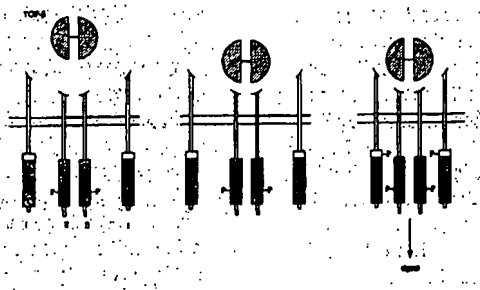

Figure 4. Signaling via TGFβ Receptors

Schematic illustration of the mechanism of signaling via TGFβ receptors as suggested by Wrana et al. (1994). TGFβ binds first to type II receptors that have a constitutively active serine/threonine kinase. The type I receptor is then incorporated in the complex and activated by phosphorylation in the GS box. Serine/threonine kinase domains are stippled, and GS boxes are open.

residues (GS domain) in their cytoplasmic juxtamembrane domains. Both receptors are needed for signaling (Wrana et al., 1992), and the cytoplasmic parts of the receptors are not interchangeable (Okadome et al., 1994). Ligand binding induces a hetero-oligomeric complex of type I and type II receptors, most likely a heterotetramer containing two receptors of each type (Yamashita et al., 1994). Studies on TGFβ-induced phosphorylation of the receptor molecules have led to an interesting model for activation of the receptors (Wrana et al., 1994; Figure 4). The type II receptor, which occurs in a dimer also in the absence of ligand (Henis et al., 1994; Chen and Derynck, 1994) and has a constitutively active kinase, first binds TGFβ. This complex then recruits the type I receptor, which can not bind ligand in the absence of type II receptor, resulting in the phosphorylation of the type I receptor on serine residues in the GS domain. The phosphorylation presumably activates the type I receptor kinase that now can act on downstream components in the signal transduction pathway. Other members in the TGFβ family also form heteromeric complexes containing different members of the type I and type II receptor subfamilies. Thus, sequential phosphorylation between the type II and type I receptors may be a general mechanism of receptor activation of members of the serine/threonine kinase receptor family.

Is Dimerization Sufficient for Activation?

There are several examples in which activation of receptors occurs after dimerization or oligomerization induced by means other than ligand binding. Many tyrosine kinase receptors, for instance, are activated after binding of antibodies, whereas Fab fragments generally are inactive. Insertion of an extra cysteine residue in the extracellular juxtamembrane region of the EGF receptor led to the formation of a constitutively active dimeric receptor (Sorokin et al., 1994). Moreover, mutated forms of many of the tyrosine kinase receptors have been identified as transforming oncogenes. In some cases, the activating mechanism is a gene rearrangement that leads to the production of a fusion protein between a novel protein and the kinase domain of the receptor. The fusion partners are often domains of proteins that undergo oligomerization in their normal context. Examples include tropomyosin, which has been found fused to Trk (Martin-Zanca et al., 1986), the regulatory subunit of the cyclic AMP-dependent protein kinase, which has been found fused to Ret (Takahashi et al., 1985), and sequences from Tpr, containing a leucine zipper, which has been found fused to Met (Park et al., 1986; Rodrigues and Park, 1994) as well as to Trk (Greco et al., 1992). Another mechanism is exemplified by the Neu (ErbB2) oncogene product, which obtained transforming activity by a single amino acid exchange in the transmembrane region that promotes receptor aggregation (Weiner et al., 1989). In these cases, artificially induced receptor dimerization leads to activation of the kinase domains and autophosphorylation in a ligand-independent manner.

Also cytokine receptors can acquire transforming properties after mutation. A constitutively active EPO receptor mutant was found to have an arginine residue replaced with a cysteine residue in a region corresponding to the receptor dimer interface of the related GH receptor; this resulted in the formation of a disulfide bond that stabilized the receptor dimer in a ligand-independent manner (Watowich et al., 1992). This finding further supports the concept that dimerization is sufficient for activation of many receptor types.

Antagonists

There are many examples of tyrosine kinase receptors and cytokine receptors that after mutations in their cytoplasmic domains act in a dominant negative manner, i.e., when expressed in cells with the corresponding wild-type receptor, they attenuate the signals induced by ligands. The mechanism for the dominant negative effect is that the wild-type receptors after ligand binding are locked up in sterile heteromeric complexes with the mutated receptors. These findings provide support for the notion that dimerization of wild-type receptors is necessary for activation of many receptor types; however, alternative modes for activation have not been excluded. Another way in which the oligomerization process can be antagonized is through mutated versions of certain ligands. For example, mutation of one of the two receptor-binding sites in GH yielded a GH protein with antagonistic properties (Fuh et al., 1992). Moreover, mutation of a glutamic acid residue in GM-CSF (Glu-21) that is important for the interaction with the common β subunit (Hercus et al., 1994), mutation of Tyr-124 in IL-4, which is important for interaction with the common γ subunit (Kruse et al., 1992), or mutation of Tyr-31 and Gly-35 in IL-6, which are important for interaction with the gp130 signal transducer (Savino et al., 1994), yielded molecules with antagonistic effects in their respective systems.

It is possible that inhibition of receptor oligomerization is a generally applicable method to antagonize growth factor and cytokine action. Antagonistic ligands and antibodies may have particular clinical utility in conditions of overactivity of growth factors and cytokines, since they can act specifically.

Conclusions

It is now well established that several receptor types are activated through ligand-induced receptor dimerization or oligomerization. Dimerization combines accuracy with flexibility; there is specificity in binding of the ligand to the receptors and flexibility in the assembly of different homo- or heterodimeric receptor subunits depending on which receptors and signal transducers are expressed by a particular cell. There are also examples of receptors that do not dimerize after ligand binding, e.g., the serpentine receptor family, which transverses the cell membrane seven times and couples to G proteins, and ion channel receptors. However, for receptor molecules that are anchored in the membrane with a single transmembrane domain, dimerization or oligomerization may be a general mechanism for receptor activation.

A general feature of receptors generating growth stimulatory signals seems to be activation of tyrosine kinases in the receptor complex. Although the exact mechanisms for activation of the kinases remain to be elucidated, interactions and cross-phosphorylations between identical or related kinases induced by receptor dimerization are common. The resulting phosphorylations of tyrosine residues on receptor and signal transducing components trigger interactions with SH2-containing molecules (see Cohen et al., 1995). Growth inhibitory signals from the activated TGFβ receptor complex involve phosphorylation on serine/threonine residues in yet unknown substrates. Thus, much of intracellular signaling is regulated by phosphorylation events. To understand the regulation of signal transduction, it will therefore be important to characterize not only the kinases involved, but also the phosphatases that counteract the effects of kinases (see Hunter, 1995 [this issue of *Cell*]).

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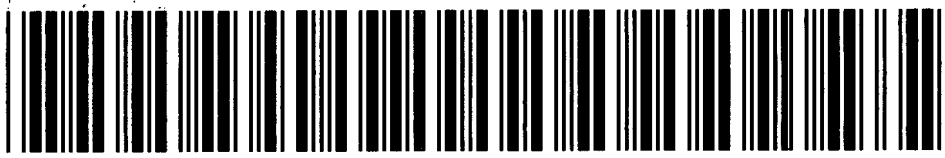

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Coexpression of *erb*B2 and *erb*B3 Proteins Reconstitutes a High Affinity Receptor for Heregulin*

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The heregulin/*neu* differentiation factor gene products were purified and cloned based on their ability to stimulate the phosphorylation of a 185-kDa protein in human breast carcinoma cell lines known to express *erb*B2. However, not all cells that express *erb*B2 respond to heregulin, indicating that other components besides *erb*B2 may be required for heregulin binding. Cells that are transfected with the closely related receptor, *erb*B3, display a single class of lower affinity heregulin binding sites than has been previously observed on breast carcinoma cell lines. Little or no stimulation of tyrosine phosphorylation in response to heregulin occurs in cells that are transfected with *erb*B3 alone. Transfection of cells with *erb*B3 and *erb*B2 reconstitutes a higher affinity binding receptor, which is also capable of generating a tyrosine phosphorylation signal in response to heregulin. A monoclonal antibody to *erb*B2 will inhibit heregulin activation of tyrosine phosphorylation and binding in cells transfected with both receptors but not with *erb*B3 alone. In cells expressing *erb*B2 and *erb*B3, both proteins become tyrosine-phosphorylated upon interaction with heregulin. Direct interaction between heregulin and the two proteins was demonstrated by chemical cross-linking experiments using ^{125}I-heregulin followed by immunoprecipitation with antibodies specific for *erb*B2 or *erb*B3.

Several diverse biological activities have recently been attributed to a family of related proteins that can arise from alternative RNA splicing of a single gene product (1). Included in this family are the heregulins (HRG)[1] (2), *neu* differentiation factor (3), acetylcholine receptor inducing activity (4), and glial growth factor. The HRGs and *neu* differentiation factor (the rat homologue of HRGα) (5) were originally purified based on their ability to stimulate the phosphorylation of a 185-kDa protein that was expressed in a number of breast carcinoma cell lines. This 185-kDa protein was shown to be p185^{erbB2}, also known as HER-2 or *neu*, based on the observation that a tyrosine-phosphorylated form of p185^{erbB2} could be immunoprecipitated from cell lines following HRG treatment. Furthermore, a radiolabeled EGF-like domain of HRGβ1 (residues 177–244) can be chemically cross-linked to a 180–185-kDa protein on breast carcinoma cell lines and immunoprecipitated with anti-p185^{erbB2} antibodies (2). These data argued that *erb*B2 is a target/receptor for HRG. However, additional studies revealed that HRG binding and activation of p185^{erbB2} was not always observed in cells that expressed *erb*B2 (6). Thus, these observations indicated that another cellular component, perhaps closely related to *erb*B2, was necessary for conferring full HRG responsiveness. Recently, we obtained data suggesting that the *erb*B3 (HER-3) protein is a candidate for such a component (7). Specifically, a single class of HRG binding sites was observed when bovine *erb*B3 was expressed in insect cells using a baculovirus expression system. However, the affinity for the HRG-*erb*B3 binding interaction (K_D ~ 0.9–3.0 nM) was significantly weaker than that measured for the binding of HRG to breast carcinoma cell lines (K_D ~ 0.1 nM) (2). Moreover, no increase in *erb*B3 protein tyrosine phosphorylation was observed upon HRG treatment of insect cells expressing *erb*B3. These findings, together with those implicating *erb*B2 as a target for HRG, raised the possibility that *erb*B3 may work together with *erb*B2 to provide a high affinity receptor for HRG with tyrosine kinase capability. The present study addresses whether direct interaction between HRG and p185^{erbB2} can be mediated by *erb*B3 and if p185^{erbB2} is required to generate a phosphorylation signal.

EXPERIMENTAL PROCEDURES

Antibodies to erbB2 and erbB3 Proteins—The anti-p185^{erbB2} monoclonal antibodies 3E8, 2C4, and 2H11 have been described previously (8). Western blot analysis for p185^{erbB2} was performed using the anti-*c-neu* monoclonal antibody, Ab-3, obtained from Oncogene Sciences. Western blot analysis for the *erb*B3 protein was performed using an anti-*erb*B3 polyclonal (rabbit) antibody obtained from Transduction Laboratories. Antibodies to the synthetic peptide ELEPELDLDLDLDLE, corresponding to residues 986–998 of the human *erb*B3 protein, were raised in rabbits and affinity-purified essentially as described by Prigent *et al.* (9).

Recombinant Heregulins—The data presented here were generated using the 68-amino acid peptide corresponding to the EGF-like domain of HRGβ1. The EGF-like domain of HRGβ1 corresponding to residues 177 to 244 was expressed in *Escherichia coli* and purified to homogeneity as described previously (2). We have verified that the full-length versions of HRGβ1 and HRGα produced and purified from mammalian cells both bind to *erb*B3 and exhibit the high affinity binding elicited by the presence of *erb*B2 together with *erb*B3. Thus, in agreement with previous observations (2), the EGF-like domain appears to be sufficient for HRG binding and the specificity of binding is not detectably α or β1 isoform-specific in this system.

Expression Vectors and Transfections—The cDNA encoding bovine *erb*B3 was subcloned into the pRK7 expression vector containing the cytomegalovirus promoter (10). The cDNA encoding human *erb*B2 also in pRK7 was obtained from Sharon Erickson, Genentech, Inc. COS-7

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[1] The abbreviations used are: HRG, heregulin; r, recombinant; EGF, epidermal growth factor.

cells (ATCC CRL 1651) were transfected using the LipofectAMINE™ protocol obtained from Life Technologies, Inc. Cells were transfected in 15-cm plates using a total of 18 μg of DNA. When cells were transfected with both *erb*B2 and *erb*B3, 9 μg of each expression plasmid was used. Single transfections were performed using 9 μg of the specific cDNA-containing plasmid and 9 μg of the pRK7 vector control plasmid. Transfections were carried out for 24–36 h, and experiments were initiated immediately.

^{125}I-HRG Binding Assay—Purified rHRG was iodinated using the lactoperoxidase method, and the radiolabeled protein was then purified by reversed phase high performance liquid chromatography using a C4 column. The average specific activity of the radiolabeled, purified protein was 300 μCi/μg. Cells were removed from 15-cm dishes 24–36 h after transfection using 2 mM EDTA in phosphate-buffered saline. Binding reactions were performed on ice for 12–16 h using 1–3 × 10^5 cells/incubation. Cell-bound ^{125}I-rHRGβ1 was separated from free by centrifugation through silicon-paraffin oil (11). Data were analyzed using a nonlinear regression program (12).

Tyrosine Phosphorylation Assay—For tyrosine phosphorylation assays, COS-7 cells were transfected in 12-well plates. Cells were stimulated with the indicated concentrations of HRG for 8 min at room temperature. Supernatants were decanted, and reactions were stopped by the addition of 200 μl of SDS sample buffer. Samples (30 μl) were electrophoresed on 4–12% polyacrylamide gradient gels (Novex) and then electroblotted onto nitrocellulose. Blots were probed with anti-phosphotyrosine monoclonal antibody-horseradish peroxidase conjugate (Transduction Laboratories), and immunoreactive bands were visualized using enhanced chemiluminescence with reagents obtained from Amersham Corp.

Chemical Cross-linking and Immunoprecipitation—Cells (1.0 × 10^6) were suspended in Hank's balanced salts containing 20 mM HEPES, pH 7.4, and incubated for 20 min at room temperature with ^{125}I-rHRGβ1 (0.5 nM) in the presence or absence of 200 nM unlabeled rHRGβ1. Bis(sulfosuccinimidyl)suberate was then added to a final concentration of 1.0 mM, and the samples were incubated for an additional 20 min. Samples were run on SDS-polyacrylamide gels (5%), and radioactive, cross-linked complexes were visualized by autoradiography.

For immunoprecipitation experiments, COS-7 cells were transfected with *erb*B3 alone or *erb*B2 and *erb*B3 and chemical cross-linking was performed as described above, except that cells were pretreated with rHRGβ1 (200 nM), buffer 2C4 (200 nM), or control antibody 2H11 (200 nM) for 20 min at room temperature. Lysates were then prepared in 0.1% SDS, 1% Triton X-100 in Tris-buffered saline containing 10% glycerol, 1 mM EDTA, 0.5 mM Na_3VO_4, 0.2 mM phenylmethylsulfonyl fluoride, 50 trypsin inhibitory units/liter aprotinin, 10 μM leupeptin. Immunoprecipitations were performed with anti-p185^{erbB3} (3E8) or anti-*erb*B3 COOH-terminal peptide antibody. Immune complexes were purified by absorption on protein A/G-agarose (Pierce Chemical Co.). Samples were analyzed by electrophoresis on polyacrylamide gels as described above.

RESULTS AND DISCUSSION

We chose to study the potential interaction of *erb*B2 and *erb*B3 proteins using transient transfections in COS-7 cells. Northern and Western blot analyses revealed that COS-7 cells expressed very low but detectable levels of *erb*B2 (data not shown); however, as shown in Fig. 1*A*, COS-7 cells transfected with a control expression plasmid (10) showed no detectable binding of ^{125}I-rHRGβ1. When COS-7 cells were transfected with an *erb*B2 expression plasmid, no increase in HRG binding was observed. Expression of the *erb*B2 protein was confirmed by Western blot analysis of the transfected COS-7 cell lysates (data not shown). Cells transfected with *erb*B3 alone displayed a single affinity binding site (4.3 × 10^5 sites/cell) with a K_D = 1.9 ± 0.3 nM. This dissociation constant for HRG is similar to that measured for insect cells expressing *erb*B3 (7). When COS-7 cells were transfected with equal amounts of *erb*B2 and *erb*B3 expression plasmids, an increase in HRG binding affinity was observed. These cells also exhibited a single class of HRG binding sites (1.6 × 10^5 sites/cell) with a K_D for HRG binding of 0.13 ± 0.01 nM. Thus, co-transfection with both expression vectors generated a HRG binding site with similar affinity to that measured for HRG binding to the breast carcinoma cell line MCF-7 (2). Double transfections of *erb*B2 and *erb*B3 were then conducted, varying the ratio of *erb*B2 to *erb*B3 expression plas-

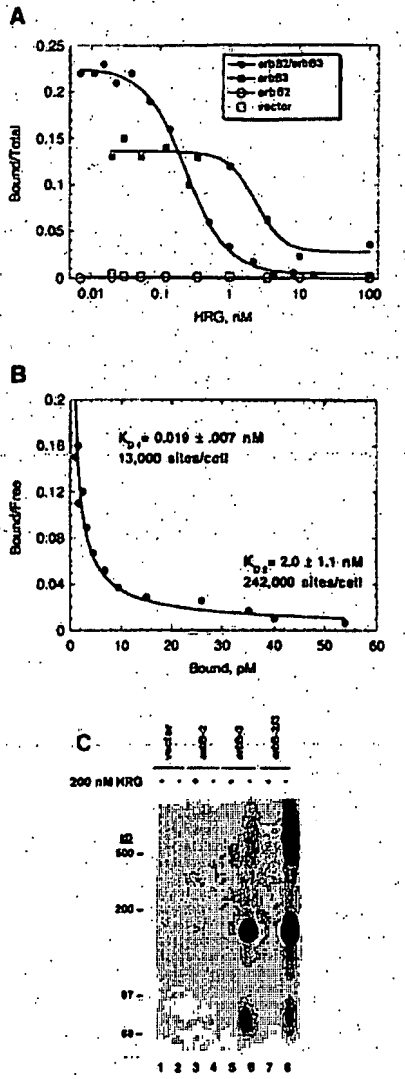

FIG. 1. **Binding of ^{125}I-rHRGβ1$_{177-244}$ to COS-7 cell transfectants.** *A*, displacement curve of ^{125}I-rHRGβ1 binding with unlabeled rHRGβ1. Cells were transfected with pRK7 control expression plasmid (□), equal amounts of *erb*B2 expression plasmid and control expression plasmid (○) (10), equal amounts of *erb*B3 expression plasmid and control expression plasmid (■), or equal amounts of *erb*B2 and *erb*B3 expression plasmids (●). *B*, Scatchard analysis of HRG binding to *erb*B2 and *erb*B3 transfectants. A 10-fold excess of *erb*B3 expression plasmid relative to *erb*B2 was used for COS-7 cell transfections. Binding experiments were performed as described in *A*. *C*, chemical cross-linking of ^{125}I-rHRGβ1$_{177-244}$ to cell surface receptors on COS-7 cell transfectants. Transfection conditions were the same as described for *panel A*. Cells (1.0 × 10^6) were detached in 2 mM EDTA, PBS and then resuspended in Hank's balanced salts containing 20 mM HEPES, pH 7.4, and incubated for 20 min at room temperature with ^{125}I-rHRGβ1 (0.5 nM) in the presence (+) or absence (−) of 200 nM unlabeled rHRGβ1. Bis(sulfosuccinimidyl)suberate was then added to a final concentration of 1.0 mM, and the samples were incubated for an additional 20 min. Samples were run on SDS-polyacrylamide gels (5%), and radioactive, cross-linked complexes were visualized by autoradiography. *Lanes 1* and *2* are COS-7 cells transfected with control expression plasmid, *lanes 3* and *4* with *erb*B2, *lanes 5* and *6* with *erb*B3, and *lanes 7* and *8* with equal amounts of *erb*B2 and *erb*B3 expression plasmids.

mids used for transfection. The total amount of expression plasmids used for transfection remained constant at all ratios throughout a given experiment. Scatchard analysis of HRG binding to cells transfected with a 10-fold excess of *erb*B3 over *erb*B2 is shown in Fig. 1*B*. The curvilinear nature of this Scatchard plot indicates the presence of two binding sites with different affinities. The high affinity sites (1.3×10^4 sites/cell) had a K_D value of 0.02 ± 0.01 nM, while the lower affinity binding sites (2.4×10^5 sites/cell) had a K_D of 2.0 ± 1.1 nM. The latter value was similar to that measured for COS-7 cells transfected with *erb*B3 alone. Increasing the amount of *erb*B2 expression plasmid to *erb*B3 expression plasmid used for transfection caused an increase in the number of high affinity binding sites. Moreover, when an expression plasmid for *erb*B3 was transfected into DHFR/G8 cells, a fibroblast cell line that overexpresses *neu* (13) but does not normally bind HRG, only high affinity HRG binding sites were observed ($K_D = 0.02$ nM; data not shown). Taken together these data indicate that, while the *erb*B3 protein alone binds HRG, a higher affinity receptor is generated when *erb*B2 is expressed together with *erb*B3.

Chemical cross-linking experiments were utilized to study the interaction of HRG with the *erb*B3 protein in the presence and absence of the *erb*B2 protein. As shown in Fig. 1*C*, HRG-specific cross-linked products in the 175–190-kDa range were observed when COS-7 cells were transfected with the *erb*B3 expression plasmid (Fig. 1*C*, *lanes 6* and *8*). No detectable cross-linked proteins were observed with the vector control or the *erb*B2 alone transfectants (Fig. 1*C*, *lanes 2* and *4*). Higher molecular mass complexes (Fig. 1*C*, *lanes 6* and *8*) were also observed when cross-linking was performed on COS-7 cells transfected with *erb*B3. This complex migrated more slowly on a 5% polyacrylamide gel than purified, recombinant apolipoprotein(a), a protein with an apparent molecular mass of 500 kDa (14). The intensity of this high molecular size complex compared to the 175–190-kDa product is greater in cells transfected with *erb*B2 and *erb*B3 than in cells transfected with *erb*B3 alone, indicating a greater propensity to form multimeric species upon HRG binding to the double transfectants.

The direct role of *erb*B2 in the formation of a high affinity HRG receptor was further addressed using a panel of monoclonal antibodies previously developed against $p185^{erbB2}$ (8). We have determined that several anti-$p185^{erbB2}$ monoclonal antibodies were capable of inhibiting HRG binding and tyrosine phosphorylation of $p185^{erbB2}$ in breast carcinoma cell lines.[3] One of these antibodies, 2C4, was tested for its ability to block HRG binding to cells transfected with *erb*B3 alone or with *erb*B2 and *erb*B3. As seen in Fig. 2*A*, HRG binding to COS-7 cells expressing both *erb*B2 and *erb*B3 was inhibited by 2C4 in a dose-dependent manner. The residual HRG binding remaining, in the presence of saturating concentrations of 2C4, was due to HRG binding to *erb*B3, since the amount of binding observed was similar to that measured in COS-7 cells expressing *erb*B3 alone. In contrast, HRG binding to COS-7 cells expressing *erb*B3 alone was not significantly inhibited by 2C4.

The identity of the cross-linked species was examined by immunoprecipitation experiments using a monoclonal antibody 3E8 directed against $p185^{erbB2}$ (8) and a rabbit polyclonal antibody raised against a synthetic peptide corresponding to residues 986–998 of the human *erb*B3 protein. Chemical cross-linking experiments were performed as in Fig. 1*C*, but cells were subsequently solubilized with 0.1% SDS and 1% Triton X-100, which dissociates non-covalent interactions between *erb*B2 and *erb*B3 (data not shown). As shown in Fig. 2*B* (*lanes 1–4*), we were not able to demonstrate a significant immunoprecipitation of protein cross-linked to ^{125}I-rHRGβ1, using the anti-*erb*B2 antibody, in COS-7 cells transfected with *erb*B3 alone. This was expected because although HRG will bind and cross-link to *erb*B3, this complex should not be precipitated by a specific anti-*erb*B2 antibody. However, the immunoprecipitation of cross-linked proteins with the anti-*erb*B2 antibody was observed in COS-7 cells transfected with both *erb*B2 and *erb*B3 (Fig. 2*B*, *lanes 6* and *8*). These data indicate that in the *erb*B2/*erb*B3 COS-7 transfectants, the ^{125}I-rHRGβ1-cross-linked complexes contain *erb*B2. Similar experiments were performed using the anti-*erb*B3 antibody (Fig. 2*B*, *lanes 9–16*). In these cases, a predominant ^{125}I-rHRGβ1 cross-linked band of ~175–190 kDa was immunoprecipitated both from cells expressing *erb*B3 alone (*lanes 10–12*) and from cells expressing *erb*B3 and *erb*B2 (*lanes 14–16*). Other than the relative intensity of the very high molecular size complex, cells expressing both *erb*B2 and *erb*B3 and immunoprecipitated with anti-*erb*B3 (*lane 14*)

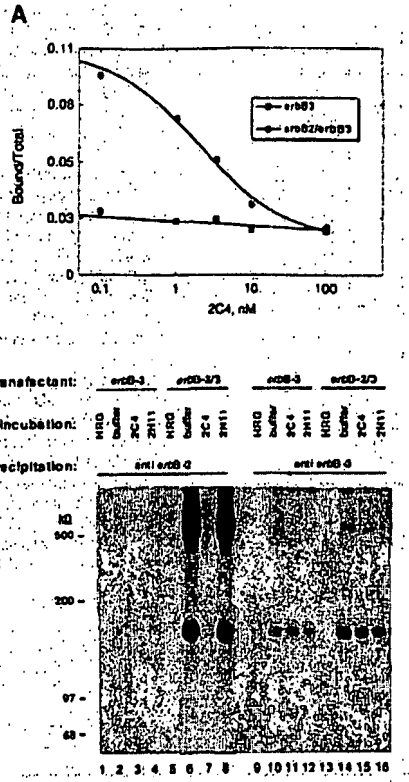

FIG. 2. **Inhibition of HRG binding by 2C4 monoclonal antibody to $p185^{erbB2}$.** *A*, COS-7 cells expressing *erb*B3 or *erb*B2 and *erb*B3 were incubated with the indicated concentrations of 2C4 on ice for 4 h. ^{125}I-rHRGβ1 (10 pM) was then added, and binding reactions were performed as described in Fig. 1*A*. Binding of ^{125}I-rHRGβ1 was 0.1% of input in the presence of 100 nM unlabeled HRG. Transfections were performed using 9 μg of *erb*B3 expression plasmid and 9 μg of control expression plasmid for *erb*B3 cells or 9 μg of *erb*B3, 0.9 μg of *erb*B2, and 8.1 μg of control expression plasmid for *erb*B2/*erb*B3 cells. *B*, effect of 2C4 on chemical cross-linking of ^{125}I-rHRGβ1 to COS-7 cell transfectants. COS-7 cells were transfected with *erb*B3 alone or *erb*B2 and *erb*B3, and chemical cross-linking was performed as described for Fig. 1*C*, except that cells were pretreated as indicated with rHRGβ1 (200 nM), buffer, 2C4 (200 nM), or control antibody 2H11 (200 nM) for 20 min at room temperature. Lysates were prepared in buffer containing 0.1% SDS, 1% Triton X-100 as described under "Experimental Procedures." Immunoprecipitations were performed with anti-$p185^{erbB2}$ (3E8) or anti-*erb*B3 COOH-terminal peptide antibody. Samples were analyzed by electrophoresis on polyacrylamide gels as described in Fig. 1*C*.

[3] M. X. Sliwkowski, J. A. Lofgren, and B. M. Fendly, unpublished data.

show a nearly identical pattern to cells expressing *erb*B3 alone. The relatively low level of high molecular size complex that is immunoprecipitated with the anti-*erb*B3 antibodies suggests that the *erb*B3 protein may be inefficiently cross-linked to itself.

After cross-linking the electrophoretic mobilities of the *erb*B2 and *erb*B3 proteins were not distinguishable in these gel systems. This is in agreement with a recent report that demonstrated the molecular size of the *erb*B3 protein is approximately 180 kDa in breast carcinoma cell lines (15). To definitively determine whether HRG was directly bound to *erb*B2, as well as to *erb*B3, we performed cross-linking experiments in the presence of an *erb*B2 antibody, 2C4, that specifically blocks HRG binding to *erb*B2 (but not to *erb*B3). To do this, the COS-7 transfectants were incubated with the 2C4 or with a control *erb*B2 antibody (2H11) that does not inhibit HRG binding, prior to incubation with ^{125}I-rHRGβ1 and chemical cross-linking reagents. The HRG cross-linked products were then immunoprecipitated with anti-*erb*B2 or anti-*erb*B3 antibodies. The results presented in Fig. 2*B* (*lane 7*) illustrate that 2C4 antibody prevented the immunoprecipitation of a HRG cross-linked band in cells that were transfected with both *erb*B2 and *erb*B3 and then immunoprecipitated with an anti-*erb*B2 antibody. No inhibition was observed with the control antibody (*lane 8*). No inhibition was observed by the 2C4 antibody of the immunoprecipitation of a HRG cross-linked band when cells were transfected with *erb*B3 alone, or with *erb*B2 and *erb*B3, when immunoprecipitations were performed with the specific anti-*erb*B3 antibody (Fig. 2*B*, *lanes 11* and *15*). These data support the specificity of the 2C4 monoclonal antibody for *erb*B2. Thus, *erb*B2 will directly bind (and cross-link) to HRG, but only when *erb*B3 is expressed in the same cell.

The HRG stimulation of receptor tyrosine kinase activity of the different COS-7 cell transfectants was examined using immunoblotting with anti-phosphotyrosine antibodies. As shown in Fig. 3*A*, cells transfected with a control expression plasmid (*lane 1* in each panel) or an expression plasmid for *erb*B2 (*lane 2* in each panel) showed no HRG-dependent increase in tyrosine phosphorylation. Cells transfected with *erb*B3 alone (*lane 3* in each panel) showed a slight phosphorylation signal in response to HRG. However, a marked HRG-dose dependent tyrosine phosphorylation signal was obtained with COS-7 cells expressing both *erb*B3 and *erb*B2 (*lane 4* in each panel).

To further investigate the role of *erb*B2 in the generation of a tyrosine phosphorylation signal, COS-7 cells that had been transfected with *erb*B3 alone or with *erb*B2 and *erb*B3 were incubated in the presence of the anti-*erb*B2 antibody, 2C4, and then assayed for HRG-dependent tyrosine phosphorylation activity. As seen from Fig. 3 (*panel B*), incubation with 2C4 significantly decreased the HRG-stimulated tyrosine phosphorylation in the *erb*B2/*erb*B3 transfectants. Although the *erb*B3 protein is capable of binding HRG alone, this binding event does not appear to generate a strong tyrosine phosphorylation signal. There are other reasons to question whether *erb*B3 is capable of autophosphorylation or tyrosine kinase activity. Foremost among these is that the comparison of the sequences of a number of protein kinases indicates that *erb*B3 differs from all other kinases at four positions; particularly noteworthy is the presence of an asparagine residue at position 834 of *erb*B3, which is an aspartic acid in all other kinases and which is thought to be an essential residue in the active center of protein kinase A (16–18). Similar amino acid substitutions when present in *kit* (19) or *fps* (20) result in a protein that is kinase-defective. It is perhaps significant that the carboxyl-terminal domain of *erb*B3 contains 13 potential phosphorylation sites (21, 22), whereas other members of this receptor family contain four or five. Thus, even if *erb*B3 is incapable of autophosphorylation or tyrosine kinase activity, it may be phosphorylated by other receptor tyrosine kinases and perhaps recruit other SH-2-containing proteins to the membrane.

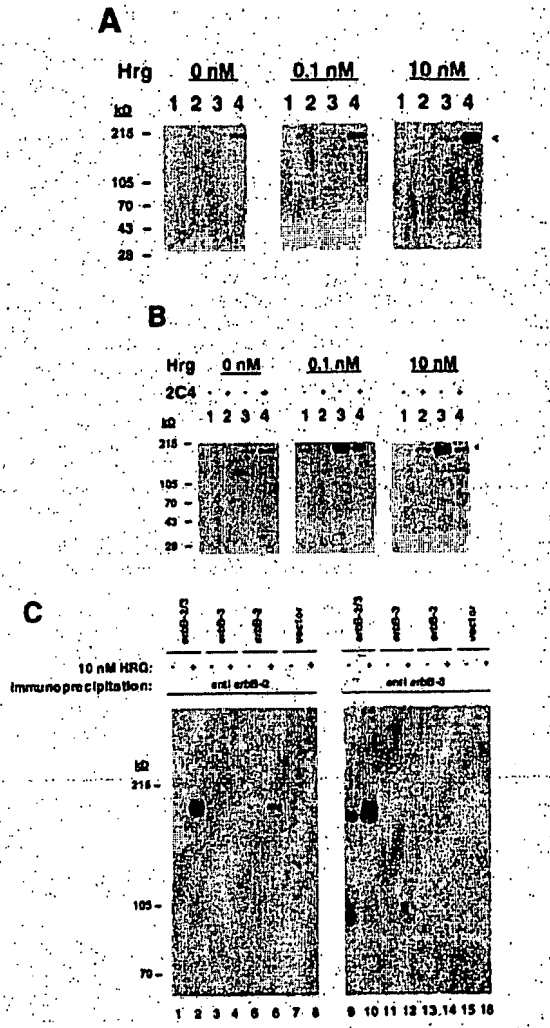

FIG. 3. **HRG stimulation of tyrosine phosphorylation.** *A*, COS-7 cells in 12-well plates were transfected with control expression plasmid (*lane 1*) or *erb*B2 alone (*lane 2*), *erb*B3 alone (*lane 3*), or *erb*B2 and *erb*B3 (*lane 4*, 10-fold excess of *erb*B3 expression plasmid over *erb*B2 was used for transfection). *B*, inhibition of tyrosine phosphorylation by anti-p185^{erbB2} monoclonal antibody 2C4. COS-7 cells transfected as described in *A* with *erb*B3 alone (*lanes 1* and *2*) or *erb*B2 and *erb*B3 (*lanes 3* and *4*) were incubated first with 2C4 (110 nM, *lanes 2* and *4*) for 30 min at room temperature and then with the indicated concentrations of HRG for 8 min. Blots were probed with anti-phosphotyrosine and developed as described for *panel A*. *C*, immunoprecipitation of HRG-stimulated COS-7 cells. COS-7 cells transfected with *erb*B2 and *erb*B3 (as described in *A*) were incubated in the presence (+) of rHRG (10 nM) or control buffer (–). Reactions were conducted as described in *A* except that samples were immunoprecipitated as described in *B* with anti-p185^{erbB2} (3E8) or anti-*erb*B3 COOH-terminal peptide antibody and then electrophoresed and immunoblotted with anti-phosphotyrosine monoclonal antibody.

To determine whether tyrosine phosphorylation occurred on one or both of these proteins when they were complexed with HRG, immunoprecipitation experiments were performed on lysates from HRG-treated cells using the specific anti-*erb*B2 or anti-*erb*B3 antibodies. COS-7 cells transfected with both the *erb*B2 or *erb*B3 expression plasmids were first stimulated with

HRG, precipitated with anti-*erb*B2 and anti-*erb*B3 antibodies, and then the precipitates were Western blotted with anti-phosphotyrosine antibody. The data presented in Fig. 3*C* show that both *erb*B2 (*lane 2*) and *erb*B3 (*lane 10*) are recognized by the anti-phosphotyrosine antibody in a HRG-stimulated manner. This type of dual phosphorylation could occur through a number of possible mechanisms. For example, HRG binding to *erb*B2 within a *erb*B2-*erb*B3 complex could stimulate *erb*B2 to undergo an intramolecular autophosphorylation event as well as to trans-phosphorylate *erb*B3. It is also possible that, although *erb*B3 alone shows little if any tyrosine kinase activity, the presence of a high local concentration of a potential substrate, *erb*B2, within an *erb*B2-*erb*B3 complex may enable the weak kinase (*erb*B3) to trans-phosphorylate *erb*B2. A somewhat similar situation has been described by Spivak-Kroizman *et al.* (23), who studied the interaction of a kinase negative point mutation of EGF receptor and its interaction with *erb*B2.

In summary, we show that the formation of a HRG-stimulated *erb*B2-*erb*B3 interaction provides for a potentially novel signaling system, where one of the participants (*erb*B3) alone has little or no tyrosine kinase activity, but when expressed with *erb*B2, contributes to a high affinity growth factor binding site and provides unique tyrosine residues, which, when trans-phosphorylated, may recruit specific signaling molecules. This differs from other interactions that have been characterized such as EGF-stimulated EGF receptor-p185^{erbB2} complex (24–27), and the more recently reported *erb*B2-*erb*B4 (HER4) complex (28), where in both cases, each of the protein components making up the heterodimer is a fully active tyrosine kinase and capable of autophosphorylation. Nonetheless, given the potential for a number of different HRG-stimulated heterodimeric complexes, it may be that these different combinations are partially responsible for the pleiotropic biological activities that have been associated with the large number of HRG/*neu* differentiation factor/acetylcholine receptor inducing activity/glial growth factor gene products.

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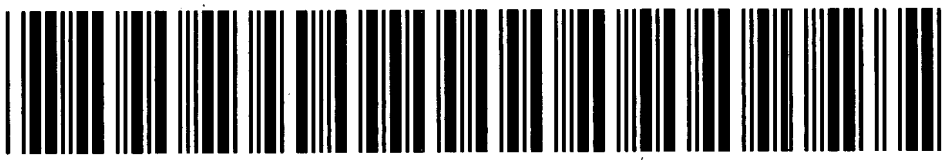

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Heregulin (HRG)-induced Mitogenic Signaling and Cytotoxic Activity of a HRG/PE40 Ligand Toxin in Human Breast Cancer Cells[1]

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Abstract

The heregulins (HRGs) are a family of growth factors that bind directly to erbB3 and erbB4 and induce tyrosine phosphorylation of erbB2 via receptor heterodimerization. Since erbB2, erbB3, and erbB4 (erbB2–4) are often overexpressed in human breast cancer cells, we produced recombinant HRGs and a HRG-based ligand toxin to investigate the signaling events triggered by HRGs and the ability of these ligands to specifically target such cells. Recombinant HRGβ2 stimulated the tyrosine phosphorylation of erbB2–4 in ZR-75-1 human breast cancer cells. This was accompanied by the tyrosine phosphorylation of Shc and the formation of complexes between Shc and the adapter protein Grb2. Complexes were also detected between Shc and erbB2–4. However, Grb2 was detected in erbB2 and erbB4 but not erbB3 immunoprecipitates. Thus, these receptors exhibit mechanistic differences in their coupling to Ras signaling, and HRGβ2 administration triggers multiple inputs into the Ras signaling pathway, involving receptor-Grb2, receptor-Shc, and Shc-Grb2 complexes. HRGβ2 addition also stimulated the association of erbB3 with phosphatidylinositol-3-kinase. In accordance with the activation of key mitogenic signaling pathways, HRGβ2 stimulated the proliferation of MCF-7 and T-47D human breast cancer cells. Moreover, when tested for the ability to stimulate cell cycle re-entry of T-47D cells arrested under serum-free conditions, HRGβ2 was more effective than insulin, previously the most potent mitogen identified using this system. Finally, a HRGβ2/PE40 ligand toxin was constructed and found to exhibit cytotoxic activity against human breast cancer cells overexpressing erbB3 alone or in combination with erbB4 and/or erbB2.

Introduction

Currently, the erbB family of RTKs[3] consists of four members: the EGFR (or erbB1), erbB2, erbB3, and erbB4 (erbB2–4). All of these receptors are monomeric in structure and belong to the class I family of RTKs (1). Several lines of evidence support a role for the erbB family of RTKs in the growth and progression of human breast cancer. For example, several EGFR ligands, including EGF, transforming growth factor α, and amphiregulin, are mitogenic for human breast cancer cells *in vitro* and are thought to function in an autocrine and/or paracrine mode to stimulate the growth of such cells *in vivo* (2). In addition, high expression of the EGFR in breast tumors is inversely related to estrogen receptor status and has been proposed as a predictor of early recurrence and death (3). Furthermore, amplification and/or overexpression of the *erbB2* gene occurs in approximately 20% of human breast cancers and is associated with decreased patient survival (4), whereas high expression of the *erbB3* and *erbB4* genes has been detected in human breast cancer cell lines (5–7). Finally, transgenic models of mouse mammary tumorigenesis support the involvement of the erbB family of RTKs and their ligands in the pathogenesis of breast cancer (8–10).

Although related in structure, members of the erbB family exhibit differences in their coupling to specific intracellular signaling pathways. For example, the EGFR and erbB2 recruit Ras GTPase-activating protein, phospholipase C-γ1, and Grb2 but exhibit only weak activation of PI3-kinase (11–14). However, despite triggering these pathways to similar extents, the erbB2 receptor exhibits a transforming potential 100-fold higher than the EGFR (15), and only the EGFR can abrogate interleukin 3 dependence in 32D hematopoietic cells (16). In the case of erbB3, qualitative differences in signaling potential have been identified. This receptor couples 10 times more efficiently to PI3-kinase than the EGFR, binds Shc, but fails to bind or tyrosine phosphorylate phospholipase C-γ1 or Ras GTPase-activating protein (17). These findings are further complicated by the observation that certain members of the erbB family can heterodimerize and transphosphorylate upon activation, leading to receptor "cross-talk." Hence, in the human breast cancer cell line SK-BR-3, activation of the EGFR leads to tyrosine phosphorylation of erbB2 (18), and in cell lines co-expressing the EGFR and erbB3, the EGFR can transphosphorylate the latter receptor, promoting the recruitment of PI3-kinase (19, 20).

Until recently, mechanistic studies on the role of erbB2–4 in human breast cancer have been hampered by the incomplete characterization of ligands for these recep-

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[3] The abbreviations used are: RTKs, receptor tyrosine kinases; EGFR, epidermal growth factor receptor; erbB2–4, erbB2, erbB3, and erbB4; PI3-kinase, phosphatidylinositol-3-kinase; HRG, heregulin; PE, *Pseudomonas* exotoxin; PE40, M, 40,000 modified PE; MBP, myelin basic protein.

tors. However, the recent cloning of the HRG/neu differentiation factor family of polypeptides (21–24) has provided the opportunity to investigate the signaling events triggered by these receptors. The HRGs are the products of a single gene and are initially synthesized as transmembrane precursors, with the extracellular region containing an immunoglobulin homology unit and an EGF-like domain, the latter being responsible for biological activity. The four HRG isoforms originally identified (HRGα, β1, β2, and β3) differ in the COOH-terminal portion of the EGF-like domain and the adjacent juxtamembrane stretch. Although erbB2 was originally proposed to represent the natural receptor for these ligands, the HRGs instead bind directly to erbB3 and erbB4, the observed tyrosine phosphorylation of erbB2 following HRG binding occurring as a consequence of heterodimerization with these receptors (25–28).

The biological effects of the HRGs on human breast cancer cells remain controversial. Several reports have described HRG-induced phenotypic differentiation of human breast cancer cells overexpressing erbB2, including altered morphology and increased synthesis of milk components including casein and lipids. These changes were accompanied by growth inhibition and induction of DNA polyploidy (21, 29, 30). However, others have observed only stimulation of proliferation of such cells (24). The reason behind these discrepancies is unclear. One problem inherent in the use of cell lines overexpressing erbB2 in the study of cellular events initiated by the HRGs is the constitutive activation of this receptor and key signaling pathways downstream of this receptor in such cells (12). Since erbB3 and erbB4 represent the primary receptors for the HRGs, an alternative approach is to use cell lines expressing more moderate levels of erbB2 in combination with these receptors, for example, MCF-7, T-47D, and ZR-75-1 human breast cancer cells. The lower baseline of receptor activation in these cell lines allows the interaction between erbB2–4 in the stimulation of key signaling pathways to be analyzed.

We have, therefore, used recombinant HRGs to examine erbB receptor activation in cell lines expressing erbB2–4 and to delineate the mechanisms underlying coupling to the Ras and PI3-kinase signaling pathways. The potent mitogenic effects of the HRGs on these cell lines are also described. Finally, we have investigated whether the levels of expression of erbB3 and erbB4 found in human breast cancer cells that overexpress these receptors, as opposed to erbB2, are sufficient to confer sensitivity to a chimeric HRG-based ligand toxin.

Results

Production of Recombinant HRG. cDNA fragments encoding the EGF-like domains of each of the four isoforms of HRG were amplified by reverse transcription-PCR and subsequently expressed in *Escherichia coli* using the pFLAG expression system (31). A ligand toxin construct encoding a fusion protein where the M_r 23,000 cell binding domain of PE (32) was replaced with HRGβ2 to produce HRGβ2/PE40 was also assembled and expressed using this system. Recombinant protein was detected in the cell fractions isolated for each HRG isoform (α, β1, β2, β3) and HRGβ2/PE40 at the predicted relative molecular weights of approximately M_r 7,000 and M_r 52,000 (the PE40 portion of the molecule actually resolves at M_r 45,000 upon SDS-PAGE), respectively (Fig. 1). Using an M1 anti-FLAG antibody affinity column, recombinant protein expressed in the periplasmic space was purified to a single band by SDS-PAGE analysis (Fig. 1*C*; only HRGβ2 and HRGβ2/PE40 shown). Yields of 0.1–1 mg/liter of culture were obtained for soluble protein purified from the periplasmic space. Upon affinity purification, the highest yield was consistently obtained with HRGβ2, and this isoform was used for the subsequent signaling and proliferation studies, as well as the production of a ligand toxin.

HRG Activation of erbB Receptors in Human Breast Cancer Cells. The ability of the HRG isoforms and the ligand toxin HRGβ2/PE40 to activate erbB2–4 was measured by the increase in tyrosine phosphorylation of a M_r 180,000–185,000 band (p180–185) detected in MCF-7 cell lysates using an antiphosphotyrosine monoclonal antibody (Fig. 2). The mobility of this band corresponds to that of erbB2–4; see for example Fig. 3. MCF-7 cells were used because they exhibit a low basal level of p180–185 tyrosine phosphorylation. The induction of p180–185 tyrosine phosphorylation exhibited a similar dose response over the concentration range 5 pM to 10 nM for each HRG isoform tested, with the addition of 10 nM HRG resulting in an approximately 10-fold increase over basal levels (Fig. 2*A*). Further assays demonstrated that the recombinant HRGs also induced tyrosine phosphorylation of p180–185 in SK-BR-3 and MDA-MB-453 human breast cancer cells at concentrations of 20 pM or greater (data not shown). EC_{50}s of 100–250 pM were observed in each cell type tested. These results compare favorably with the EC_{50} found in a previous study (EC_{50} = 40 pM) for MCF-7 cells (24). The ability of HRGβ2/PE40 to stimulate tyrosine phosphorylation of p180–185 was indistinguishable from that of HRGβ2 in MCF-7 cells (Fig. 2*B*). This demonstrates that fusion of PE40 to the HRGs does not affect the activity of these ligands as judged by their ability to stimulate p180–185 tyrosine phosphorylation.

To ascertain which erbB receptors were tyrosine phosphorylated in response to HRG addition, ZR-75-1 human breast cancer cells were used, since these cells express readily detectable levels of erbB2–4. Western blotting of erbB2 and erbB3 immunoprecipitates from ZR-75-1 cells with an antiphosphotyrosine monoclonal antibody revealed a marked increase in tyrosine phosphorylation of these receptors upon HRGβ2 (10 nM) administration (Fig. 3). Since the anti-erbB4 antibody used in these studies is only suitable for Western blotting, the reciprocal experiment was performed in which antiphosphotyrosine immunoprecipitates from control and HRG-stimulated cells were blotted with the anti-erbB4 antibody. HRG addition clearly resulted in increased recruitment of erbB4 into the antiphosphotyrosine immunoprecipitate (Fig. 3). With prolonged exposure of ECL autoradiographs, it was evident that there was a low basal level of tyrosine phosphorylation on each of erbB2–4. Therefore, these results demonstrate that erbB2–4 become tyrosine phosphorylated in ZR-75-1 human breast cancer cells upon HRG treatment. Immunoprecipitation of erbB2 and erbB3 and blotting with the same antibody served as controls (Fig. 3; only data for erbB2 shown). The same experiments were performed using cell lysates of MCF-7 breast cancer cells. Similar results were obtained except that insufficient levels of erbB4 precluded detection of activation by HRGβ2 (data not shown).

Activation of the Ras Signaling Pathway in Response to HRG-induced erbB Receptor Activation. Activation of the Ras signaling pathway by the EGFR involves recruitment of a complex between the Grb2 adapter protein and the Sos

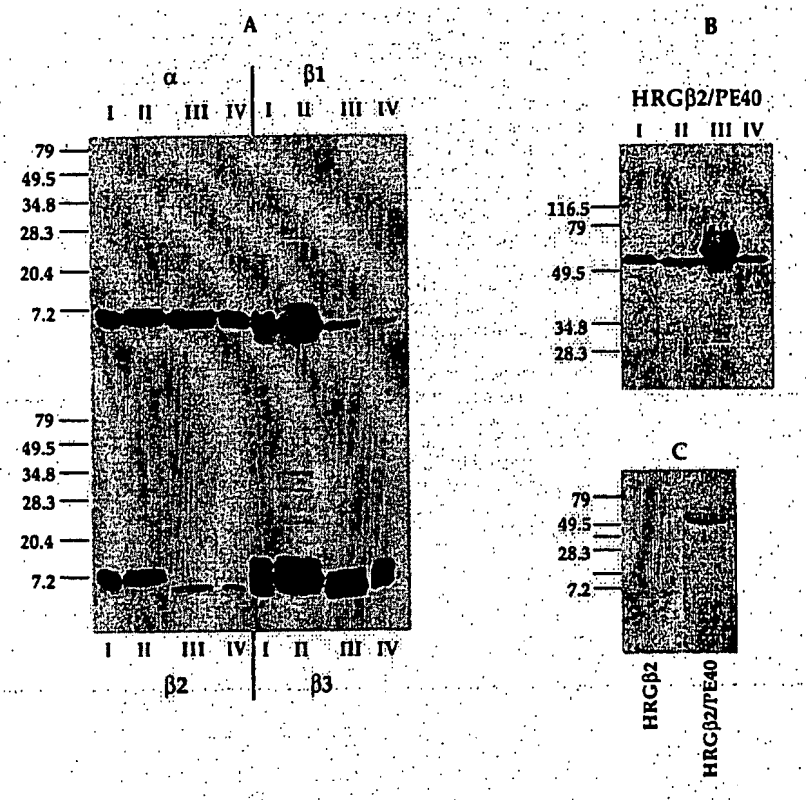

Fig. 1. Expression and purification of recombinant HRGs and HRGβ2/PE40 ligand toxin. *A* and *B*, detection of recombinant HRGs and HRGβ2/PE40 in *E. coli* cell fractions. Soluble and insoluble extracts were isolated, and the fusion protein content was evaluated by Western blot using the anti-FLAG M2 antibody. *Lane I*, whole-cell extract; *Lane II*, insoluble cytoplasmic extract; *Lane III*, soluble cytoplasmic extract; *Lane IV*, periplasmic extract. Also indicated is the isoform of HRG (HRGα, β1, β2, or β3) or the ligand toxin (HRGβ2/PE40) expressed in each culture. *C*, affinity purification of HRGβ2 or HRGβ2/PE40. Five μg of purified protein were subjected to SDS-PAGE and then stained with Coomassie Blue. Migration of molecular weight standards was as indicated; sizes are in kilodaltons.

GDP-GTP exchange factor to the tyrosine-phosphorylated receptor (33). An additional binding site for this complex is present on the tyrosine-phosphorylated Shc proteins, which can also be recruited by activated growth factor receptors (34–36). Therefore, we investigated the involvement of Grb2 and Shc in HRG-induced mitogenic signaling in ZR-75-1 cells.

Western blotting of Shc immunoprecipitates from control and HRG-stimulated cells with an antiphosphotyrosine antibody revealed increased tyrosine phosphorylation of the p52 Shc protein upon HRG addition (Fig. 4*A*). Also present in the Shc immunoprecipitates was a tyrosine-phosphorylated band of M_r 180,000–185,000. To identify the erbB receptors contributing to this band, the immunoprecipitates were Western blotted with specific anti-erbB receptor antibodies (Fig. 4*A*). These experiments demonstrated that the Shc proteins form complexes with erbB2–4 following HRG-induced receptor activation. Furthermore, Western blotting of the Shc immunoprecipitates with an anti-Grb2 monoclonal antibody revealed that tyrosine phosphorylation of the Shc proteins resulted in increased Shc-Grb2 complex formation (Fig. 4*A*). Also noteworthy was the detectable basal level of Shc association with erbB2 and erbB4 in the absence of HRG stimulation, presumably due to the basal level of tyrosine phosphorylation of these receptors (Fig. 4*A*).

To investigate the recruitment of Grb2 by erbB receptors following HRG administration, anti-Grb2 immunoprecipitates were blotted with specific anti-erbB receptor antibodies (Fig. 4*B*). Increased levels of both erbB2 and erbB4, but not erbB3, were detected in Grb2 immunoprecipitates following HRG addition. To confirm this finding, lysates from control and HRG-stimulated cells were incubated with a GST-Grb2 fusion protein coupled to agarose beads (11). Following washing, bound proteins were Western blotted with either anti-erbB3 or anti-erbB4 antibodies. This experiment demonstrated that HRG-activated erbB4, but not erbB3, bound to the Grb2 fusion protein (data not shown). Furthermore, blotting of erbB3 receptor immunoprecipitates with a Grb2 monoclonal antibody also did not detect any association of Grb2 with erbB3 (Fig. 4*B*). Immunoprecipitation and blotting of Shc, Grb2, and erbB3 served as controls (Fig. 4, *A* and *B*; only data for Shc and Grb2 shown).

To confirm activation of the Ras pathway in response to HRG administration, the activity of MAP kinase, which functions downstream of Ras, was investigated. MAP kinase p44 (ERK1) was immunoprecipitated from ZR-75-1 cells before and after HRGβ2 addition and then examined for its ability to phosphorylate an exogenous substrate, MBP. Densitometric analysis indicated that a 4–5-fold increase in MAP kinase activity was detected 15 min after HRGβ2 addition (Fig. 4*C*).

Thus, HRG addition to ZR-75-1 human breast cancer cells results in the association of erbB2–4 with Shc proteins, the tyrosine phosphorylation of p52 Shc, and the formation

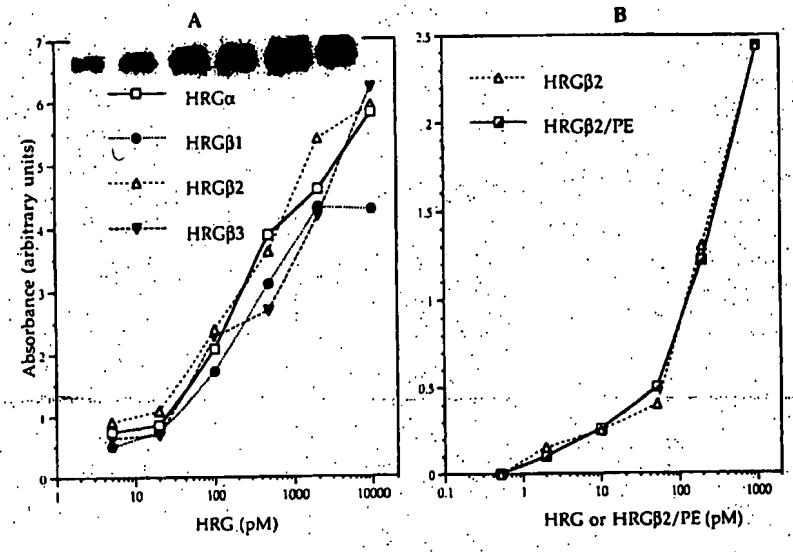

Fig. 2. Induction of p180–185 tyrosine phosphorylation by recombinant HRGs (*A*) and HRGβ2/PE40 (*B*). Monolayer cultures of the breast cancer cell line MCF-7 were incubated in 0.5% serum for 18 h before addition of recombinant proteins at the concentrations indicated. After 20 min at 37°C, the cells were lysed, and the resulting lysates were subjected to Western blot using an antiphosphotyrosine monoclonal antibody. The relative levels of p180–185 tyrosine phosphorylation were then determined by densitometric analysis of the autoradiographs. *Inset*, induction of tyrosine phosphorylation of p180–185 by HRGβ2. The concentrations of HRGβ2 used correspond to those used in the graphical representation of the data.

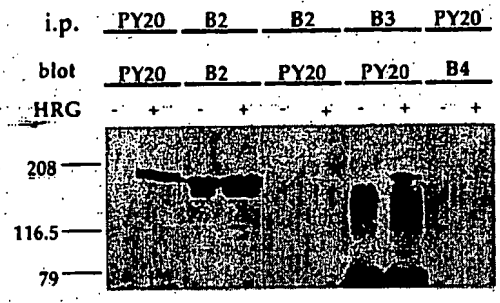

Fig. 1. HRG-induced tyrosine phosphorylation of erbB2–4 in ZR-75-1 human breast cancer cells. Cells were serum starved and then treated with a vehicle control (−) or HRGβ2 (+; final concentration, 10 nM) as indicated for 15 min at 37°C. The cells were then lysed, and the resulting lysates were subjected to immunoprecipitation (*i.p.*) and Western blot (*blot*) using the indicated antibodies. *B2*, anti-erbB2; *B3*, anti-erbB3; *B4*, anti-erbB4; *PY20*, antiphosphotyrosine. *Left*, molecular weight standards in kilodaltons.

of Shc-Grb2 complexes. Grb2 recruitment by erbB2 and erbB4 was also evident, which could be direct or mediated through the Shc proteins; clearly, these mechanisms are not mutually exclusive. However, erbB3 differs from erbB2 and erbB4 in associating with Shc proteins, but not Grb2. Thus, erbB2–4 use different mechanisms to couple to the Ras signaling pathway.

HRG-stimulated Association of PI3-kinase with erbB3. Other researchers have reported the association of PI3-kinase with activated erbB3 receptors (17, 19, 20, 37, 38). PI3-kinase is a cytosolic enzyme that is recruited to specific protein tyrosine kinases due to binding of the p85 subunit to tyrosine-phosphorylated YXXM motifs. Six such motifs are present in the cytoplasmic domain of erbB3, whereas this motif is not evident in erbB2 or erbB4 (39). Hence, we looked at whether PI3-kinase is associated with erbB3 in HRG-treated human breast cancer cells. In ZR-75-1 cells, HRGβ2 stimulated the tyrosine phosphorylation of erbB3 and increased dramatically the PI3-kinase activity associated with this receptor (Fig. 5). HRG addition also increased the co-immunoprecipitation of the p85 subunit of PI3-kinase with erbB3 (data not shown).

Effects of HRGβ2 on Human Breast Cancer Cell Proliferation. MCF-7 and T-47D human breast cancer cells, SK-OV-3 human ovarian cancer cells, and 184B5 immortalized normal breast epithelial cells were used for proliferation assays. 184B5 cells express low or undetectable levels of erbB2 and erbB3, whereas MCF-7 and T-47D cells express similar low levels of erbB2 but overexpress erbB3.[4] The latter two cell lines also express detectable levels of erbB4, as observed by PCR analysis (7) or cell-surface staining using an erbB4 antibody different to that used in this report (40). SK-OV-3 cells express both erbB2 and erbB3 receptors, but the HRGs fail to induce the tyrosine phosphorylation of erbB2 in these cells (40, 41).

The addition of HRGβ2 (0.5 pM to 5 nM) to T-47D or MCF-7 cells maintained in 5% FCS resulted in increased cell numbers relative to untreated controls with one-half maximum responses (EC_{50}) at approximately 30 pM and 300 pM, respectively (Fig. 6*A*). At 10 pM, cell numbers increased only marginally at 3, 5, and 7 days after treatment, whereas a maximum response was generally achieved at a dose of 1 nM. The effect of an optimal concentration (1 nM) of HRGβ2 on the proliferation rate of MCF-7 and T-47D cells is shown in Fig. 7. In this experiment, HRG addition to MCF-7 cells resulted in a doubling of cell numbers relative to controls after 5 days, whereas for T-47D cells, cell numbers increased by 30–40% over controls after 3–5 days (Fig. 7). Proliferation rates in HRG-treated cultures generally slowed after day 5 as the cells reached confluence. In contrast, both the SK-OV-3 and 184B5 cells showed no proliferative

[4] A. de Fazio, P. W. Janes, and R. J. Fiddes, unpublished data.

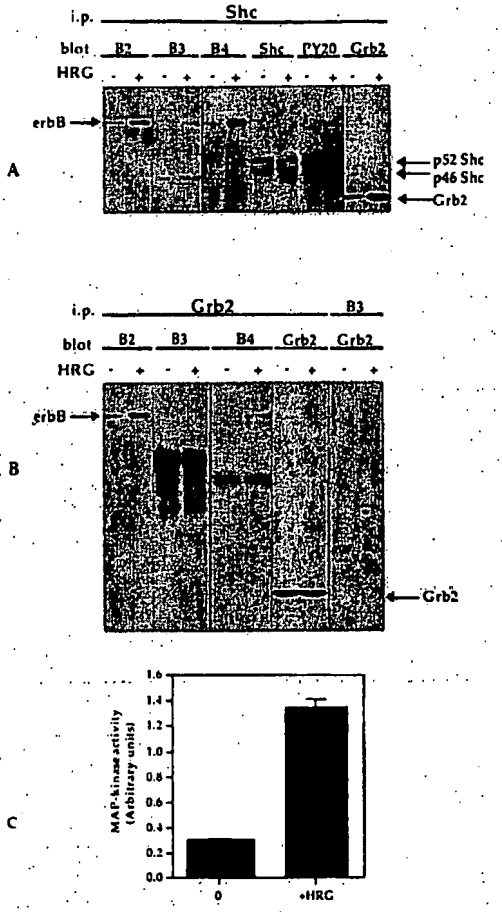

Fig. 4. Stimulation of the Ras signaling pathway by HRGβ2 in ZR-75-1 human breast cancer cells. *A* and *B*, HRG-stimulated tyrosine phosphorylation of Shc proteins and association of Shc and Grb2 (respectively) with activated erbB receptors. Monolayer cultures of cells were treated either with vehicle alone (−) or with HRGβ2 (+) as described in Fig. 3. Immunoprecipitation (*i.p.*) experiments were performed as indicated and analyzed by Western blot (*blot*) using anti-Shc, antiphosphotyrosine (*PY20*), anti-Grb2, or anti-erbB2–4 antibodies as indicated above the figure. Other bands present in the erbB3 and erbB4 blots of Grb2 immunoprecipitations are due to cross-reactivity of the secondary blotting antibody with the immunoprecipitating antibody. *C*, activation of MAP kinase in response to HRGβ2 administration. MAP kinase p44 (ERK1) was immunoprecipitated from ZR-75-1 cell lysates before (*0*) and 15 min after (*+HRG*) HRGβ2 addition (final concentration, 10 nM) and then assayed for its ability to phosphorylate an exogenous substrate, MBP. Following SDS-PAGE, radioactive bands representing phosphorylated MBP were revealed by autoradiography, and band intensities were quantified by densitometry. MAP kinase activity is expressed relative to controls following normalization for protein levels (determined by Western blotting MAP kinase immunoprecipitates for ERK1). *Bars*, SD.

responses (Fig. 6*A*); at all concentrations tested (0.5 pM to 5 nM) and at three different plating densities (200 to 1000 cells/well), there was no difference in relative cell numbers between HRGβ2-treated or untreated cells over the 7 days of the experiment.

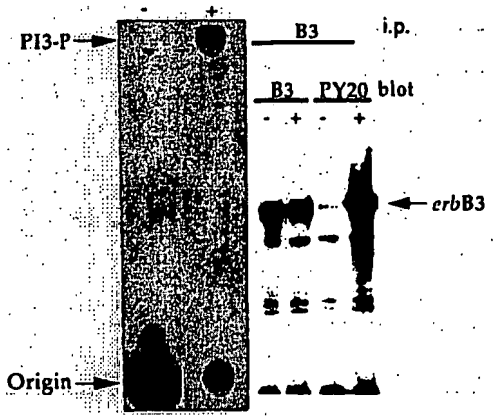

Fig. 5. HRGβ2 stimulation of erbB3-associated PI3-kinase activity in ZR-75-1 breast cancer cells. PI3-kinase activity was assayed in erbB3 immunoprecipitates from HRGβ2-treated (+) or control (−) ZR-75-1 cells, as described in "Materials and Methods." *Left panel*, autoradiograph of the reaction products following separation by TLC. *Right panel*, the result of Western blotting the immunoprecipitates with either anti-erbB3 or antiphosphotyrosine (*PY20*) antibodies.

Effects of HRGβ2 on Cell Cycle Progression in T-47D Breast Cancer Cells. Since the HRG-induced increase in cell proliferation rate occurred even in the presence of 5% FCS, we investigated the mitogenic properties of HRGβ2 under serum-free conditions. T-47D cells arrested in G_1 were treated with HRGβ2 (5 nM), and entry into S phase was monitored by DNA flow cytometry. After an approximate 12-h delay, a semi-synchronous entry of cells into S phase was observed (Fig. 6*B*), a result similar to that seen previously with other growth factor mitogens (42). In addition, the relative potencies of HRGβ2, FCS, and insulin were compared for their ability to stimulate cell cycle progression in this model. As evident from Fig. 6*C*, HRGβ2 was a more potent mitogen than either insulin or FCS.

Cytotoxicity of a HRGβ2/PE40 Ligand Toxin. Identical studies to the proliferation assays described above were performed using the ligand toxin, HRGβ2/PE40, or PE40 alone. In addition, SK-BR-3 and MDA-MB-453 human breast cancer cells were used. These cell lines overexpress (relative to normal breast epithelial cells) erbB2 and erbB3,[4] whereas MDA-MB-453 cells also overexpress erbB4 (7, 40). Each of the breast cancer cell lines treated with HRGβ2/PE40 showed a dramatic decrease in cell number, both relative to nontreated control cultures and the initial plating density (Fig. 7; only MCF-7 and T-47D data shown). Ligand toxin concentrations of greater than 2 pM generally resulted in cytotoxicity with maximum cell death seen at the highest concentration used (5 nM). After 7 days of exposure to the ligand toxin, less than 10% (relative to control cultures) of T-47D, SK-BR-3, and MDA-MB-453 cells were viable (18% for MCF-7). MDA-MB-453 breast cancer cells were the most sensitive to HRGβ2/PE40, with an IC_{50} of 7–20 pM, whereas T-47D, SK-BR-3, and MCF-7 cells were less sensitive (Table 1). When 184B5 or SK-OV-3 cells were exposed to the HRGβ2/PE40 or PE40, no significant effect on cell number was seen at any dose. At only the highest concentration of PE40 used (5 nM) was proliferation of

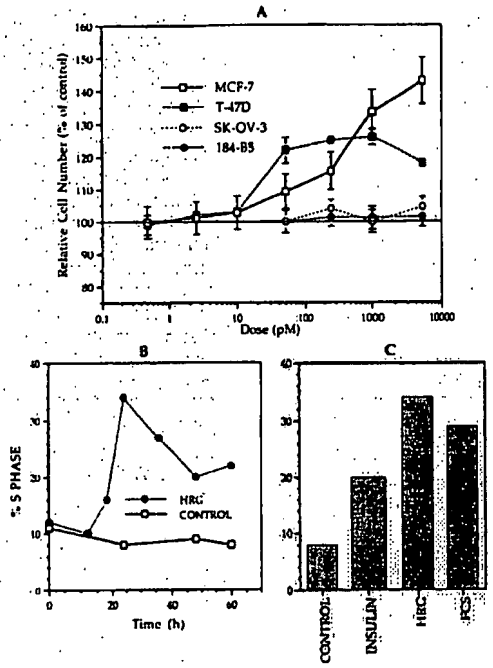

Fig. 6. Regulation of cell proliferation and cell cycle progression by HRGβ2. *A*, regulation of cell proliferation by the HRGs. MCF-7 (□) and T-47D (■) human breast cancer cells, SK-OV-3 ovarian cancer cells (○) and 184B5 immortalized breast epithelial cells (●) were plated into 96-well plates. HRGβ2 (0.5 pM–5 nM) was added at day 0, and cell numbers were evaluated using an indirect 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay at day 3. Shown are cell numbers expressed as a percentage of control untreated cells (mean of three experiments; bars, SD). *B*, regulation of cell cycle progression by HRGβ2. T-47D breast cancer cells were arrested in G_1 phase by serum starvation and then exposed to HRGβ2 (5 nM). Duplicate flasks of cells were then harvested at 12, 18, 24, 36, and 60 h, and DNA content was evaluated by flow cytometry. The graph shows the percentage of cells in S phase *versus* time for cells exposed to HRGβ2 (●) or vehicle alone (□). *C*, T-47D cells were growth arrested as above and then treated with either HRGβ2 (5 nM), insulin (10 μg/ml), or FCS (5%). The graph shows the percentage of cells in S phase 24 h after exposure to the different treatments.

MCF-7 and T-47D cells slightly retarded (Fig. 7); however, IC_{50}s for PE40 were 20–1000 fold higher than those for HRGβ2/PE40 in the breast cancer cell lines studied (Table 1).

Discussion

We describe here the production of recombinant HRG isoforms comprising the EGF-like domain that can be purified from transformed *E. coli* extracts in a single-step affinity procedure to an essentially homogenous preparation. The four HRG isoforms exhibited similar activities when assayed for their ability to stimulate p180–185 tyrosine phosphorylation in human breast cancer cells, displaying EC_{50}s of 100–250 pM. These values are comparable to those determined for recombinant $HRG\alpha_{177-239}$ and $HRG\beta1_{177-244}$ using a similar assay in MCF-7 cells; both proteins showed similar activities, exhibiting EC_{50}s of approximately 40 pM (24). Interestingly, it was observed that the β isoforms of the rat homologue of HRG, neu differentiation factor, demonstrated a 10-fold higher affinity for endogenous binding sites on T-47D cells than the α isoforms (43), and soluble extracellular domains of erbB3 and erbB4 bound the β isoforms of HRG with a higher affinity than HRGα (28). Moreover, HRGβ isoforms were more potent than HRGα in stimulating [^{3}H]thymidine incorporation and proliferation of T-47D breast cancer cells (44) and mouse keratinocytes (45). The reason for this apparent discrepancy is unclear. Recently, several reports have shown that HRGs bind to erbB3 and erbB4, with the observed tyrosine phosphorylation of erbB2 occurring as a consequence of heterodimerization (25–28). In accordance with these findings, HRGβ2 stimulated the tyrosine phosphorylation of erbB2–4 in ZR-75-1 human breast cancer cells (Fig. 3). Similar results were found in MCF-7 cells, except that the low expression of erbB4 precluded the detection of tyrosine phosphorylation of this receptor.

Ligand stimulation and subsequent activation of RTKs trigger several intracellular signaling pathways that have recently been delineated at the molecular level. Two pathways important in growth factor-stimulated mitogenesis are those leading to the activation of Ras (46) and PI3-kinase (47). SH2 domain-containing proteins, which play pivotal roles in the regulation of Ras by RTKs, are the adapter proteins Grb2 (11) and Shc (34). The SH2 domain of Grb2 binds activated RTKs containing the appropriate binding site (a strong preference is exhibited for autophosphorylation sites with asparagine at the +2 position relative to the tyrosine), whereas the SH3 domains recruit the Sos Ras GDP-GTP exchange factor. The juxtaposition of Sos next to membrane-anchored Ras is thought to lead to Ras activation (33). Both the EGFR and erbB2 form complexes with Grb2 upon activation (11, 12, 48). The Shc proteins are tyrosine phosphorylated in response to activation of many RTKs, and this phosphorylation occurs on a consensus Grb2 binding site; complex formation with Grb2 subsequently ensues (36). Although the exact function of Shc remains obscure, recent studies have highlighted the importance of the Shc-Grb2-Sos complex in Ras activation (49–51). erbB family RTKs possess two types of binding sites for Shc; NPXY motifs which, when tyrosine phosphorylated, bind a recently identified NH_2-terminal domain of Shc (the PTB, PI, or SAIN domain; Refs. 52–54) and binding sites for the SH2 domain (48, 55).

Since they express readily detectable levels of erbB2–4, ZR-75-1 human breast cancer cells provide a particularly interesting model in which to study the participation of these receptors in activation of the Ras pathway; in particular, the association of key SH2 domain-containing signaling molecules with erbB4 has not been reported previously. HRGβ2 administration resulted in the tyrosine phosphorylation of Shc and the association of this protein with erbB2–4 and Grb2. However, when Grb2 complex formation with erbB receptors was examined after HRGβ2 addition, co-immunoprecipitation with erbB2 and erbB4, but not erbB3, could be demonstrated. Our results, using native receptors, confirms that reported by Prigent and Gullick (37) using a recombinant EGFR-erbB3 chimera. This suggests that the consensus Grb2 binding sites on erbB3 (56) are either not phosphorylated, not active in binding Grb2, or inaccessible, and also, that Grb2 cannot be recruited to erbB3 via association with Shc. Our demonstration of co-immunoprecipitation of Grb2 and Shc with erbB4 is in

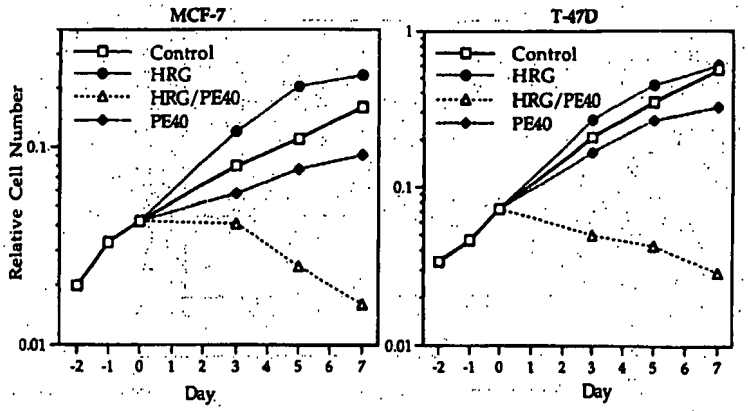

Fig. 7. Effects of HRGβ2 or HRGβ2/PE40 on the rate of proliferation of human breast cancer cells. Cells were dispensed into individual wells of 96-well culture plates. HRGβ2, HRGβ2/PE40, or PE40 (0.5 pM-5 nM) was added at day 0, and cell numbers were evaluated at days 3, 5, and 7 using an indirect 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. The graph shows typical results for doses giving the maximum response: ●, HRGβ2, 1 nM; △, HRGβ2/PE40, 5 nM; ◆, PE40, 5 nM; and □, control, vehicle alone. Each experiment was performed three or four times with essentially identical results.

Table 1 Cytotoxicity of HRGβ2/PE40 ligand toxin and the toxin component PE40 alone, 7 days after drug addition

Cell numbers are expressed relative to vehicle treated control cultures, and IC_{50}s represent the dose required for a 50% reduction in cell numbers. Presented are the results of three to four experiments for each cell line.

	HRGβ2PE40		PE40
	Relative cell number (5 nM HRGβ2/PE40, % of control ± SD)	IC_{50} (pM) (range)	IC_{50} (pM)
MCF-7	18 ± 5	300–400	~5000
T-47D	7 ± 2	50–100	~2500
SK-BR-3	6 ± 2	100–200	>5000
MDA-MB-453	5 ± 3	7–20	>5000
SK-OV-3	100 ± 0.5	>5000	>5000
184B5	100 ± 0.5	>5000	>5000

agreement with the presence of predicted binding sites for these molecules on this receptor (39, 54).

PI3-kinase consists of an *M*, 85,000 adaptor subunit (p85) and a *M*, 110,000 catalytic subunit; recruitment by activated RTKs is mediated by binding of the p85 SH2 domains to tyrosine phosphorylated YXXM motifs. Interestingly, erbB3, but not the other erbB proteins, possesses six COOH-terminal copies of this motif (39). Several groups have reported association of PI3-kinase activity with erbB3 using either EGFR-erbB3 chimeras (17, 37) or erbB3 receptors stimulated with HRGs (38) or transphosphorylated either by the EGFR (19, 20) or erbB2 (57). Our data demonstrate that HRGβ2 addition to ZR-75-1 cells leads to association of PI3-kinase activity with tyrosine-phosphorylated erbB3 (Fig. 5); this was accompanied by recruitment of the p85 subunit of the enzyme (data not shown). In addition, there was no detectable increase in PI3-kinase activity in erbB2 immunoprecipitates following HRG administration (data not shown).

Thus, the activation of erbB2–4 by HRG addition not only leads to the triggering of multiple potential inputs into the Ras pathway (*i.e.*, the formation of receptor-Grb2, receptor-Shc, and Shc-Grb2 complexes) but also recruitment of PI3-kinase to activated erbB3. We were, therefore, interested in examining the mitogenic effects of the HRGs in human breast cancer cell lines. We chose for this study two cell lines, T-47D and MCF-7, which have been well characterized for their proliferative responses to growth factors in our laboratory (42).[5] These cell lines express similar levels of erbB2 to normal breast epithelial cells but overexpress erbB3. Although MCF-7 and T-47D cells were reported to exhibit moderate and high levels of erbB4 mRNA expression, respectively (7), only low levels of this receptor were detected in these cell lines by Western and Northern blotting.[4]

In the presence of 5% FCS, MCF-7 and T-47D cell numbers increased in a dose-dependent manner, with maximum stimulation 3–5 days after HRGβ2 addition. These results are in agreement with Marte *et al.* (44), who also reported a mitogenic effect of the HRGs on T-47D cells in the presence of serum. To further define HRG-regulated cell proliferation, we studied the effects of HRGβ2 on T-47D cell cycle progression in a well-defined, serum-free model (42). In common with other breast cancer cell mitogens (for example, insulin, insulin-like growth factors, transforming growth factor α, EGF, and fibroblast growth factor), HRGβ2 stimulated cell cycle progression in growth-arrested cells, with cells entering S phase between 12 and 18 h after addition. These temporal changes were similar to those observed with the other growth factors but, more importantly, HRGβ2 represents the most potent mitogen tested in this system. The data in Fig. 6*C* demonstrate that the proportion of cells in S phase at 24 h was greater in HRG-treated cells than in cells treated with either insulin, the previously most potent individual mitogen identified, or 5% FCS. This effect on cell cycle progression is consistent with the demonstration (Figs. 6 and 7) that HRGβ2 could further increase the proliferation rate of cells growing in 5% FCS.

Our data demonstrating that HRGβ2 is a potent mitogen for MCF-7 and T-47D breast cancer cells is in marked contrast to the reported effects of this polypeptide on breast cancer cells that overexpress erbB2. In such cells (AU-565, SK-BR-3, and MDA-MB-453), HRGs apparently cause growth inhibition and induce cellular differentiation (21, 29, 30), although these data remain controversial, and in another study, only proliferative effects were observed in

[5] E. A. Musgrove, unpublished data.

SK-BR-3 cells (24). A potential explanation for these effects is that since breast cancer cells overexpressing erbB2 exhibit high levels of erbB2 tyrosine phosphorylation and MAP kinase activity (12), HRG activation of erbB3 and/or erbB4 may induce the further activation of MAP kinase or related enzymes above a threshold required for nuclear localization and triggering of a differentiation signal (58). Certainly, activation of MAP kinase and p70/p85 S6 kinase occurs in cells stimulated to either proliferate (T-47D cells) or differentiate (AU565 cells) by HRG (44). Consequently, since the HRGs are expressed by a subset of human breast cancers and breast cancer-derived cell lines (59, 60), these polypeptides are likely to function in an autocrine and/or paracrine mode to regulate breast cancer cell proliferation; however, the nature of the cellular response may depend on the relative expression levels of erbB2–4.

Finally, we have shown *in vitro* that HRGβ2, when coupled to PE40 toxin, exhibits a cytotoxic effect against human breast cancer cells that overexpress, relative to normal breast epithelial cells, erbB3 alone (T-47D and MCF-7) or in combination with erbB2 (SK-BR-3) or erbB2 and erbB4 (MDA-MB-453). These findings are in general agreement with recent publications describing similar HRG-based ligand toxins and their activity against cancer cell lines, as measured by inhibition of protein synthesis (61) or cell killing (40, 62). Significantly, we found that immortalized breast epithelial cells (184B5) were insensitive to the ligand toxin. Therefore, the HRGs represent a potential tool in the development of therapeutic strategies specifically targeting cancers that overexpress erbB3 and/or erbB4.

Materials and Methods

Antibodies. Antibodies were as follows: monoclonal anti-erbB2, Novocastra; polyclonal anti-erbB3, a gift from Dr. M. H. Kraus (NIH, Bethesda, MD), which was used for immunoprecipitations; monoclonal anti-erbB3 2F12 (19), a gift from Dr. J. G. Koland (University of Iowa, Iowa City, IA), which was used for Western blots; polyclonal anti-erbB4, Santa Cruz Biotechnology; monoclonal antiphosphotyrosine PY20, Transduction Laboratories; polyclonal anti-Grb2 Ab50 (for immunoprecipitation) (11) a gift of Dr. J. Schlessinger (New York University Medical Center); monoclonal anti-Grb2 (for Western blotting), Transduction Laboratories; polyclonal anti-Shc, UBI; and polyclonal anti-ERK antibodies K23 (for Western blotting) and 956/837 (for immunoprecipitation), Santa Cruz Biotechnology.

Cell Lines. The sources and maintenance of the human breast cancer cell lines used in this study were as described previously (63). SK-OV-3 human ovarian cancer cells were obtained from American Type Culture Collection and maintained in the same manner. 184B5 immortalized human breast epithelial cells were the kind gift of Dr Martha Stampfer (University of California, Berkeley, CA) and were maintained in mammary epithelial growth medium (Clonetics).

Amplification of cDNAs Encoding HRG Isoforms. The guanidinium isothiocyanate-cesium chloride procedure was used to isolate total RNA from the human breast cancer cell line MDA-MB-231, which expresses high levels of the HRGs. Following first-strand DNA synthesis, HRG cDNA fragments were amplified by PCR using a forward primer common to each HRG isoform (5′-GATCAAGCTTAGCCATCTTGTAAAATGTGCG-3′) containing a four-nucleotide leader sequence and a *Hin*dIII site (underlined) to facilitate subcloning, and reverse primers common to isoforms α, β1, and β2 (5′-GATCAAGCTTCTACTGGTACAGCTCCTCCGC-3′) or β3 (5′-GATCAAGCTTCTATTCAGGCAGAGACAGAAAGGG-3′) containing a leader sequence, a *Hin*dIII site (underlined), and an in-frame termination codon (double underlined). To clone cDNA suitable for the production of the HRGβ2/PE40 ligand toxin, the following reverse primer was used in which the stop codon was deleted: 5′-AGACTAAGCTTCTGGTACAGCTCCTCCGC-3′. These cDNA fragments encode the EGF-like domain of the HRGs, which has been shown previously to be the biologically active domain (24), consisting of 64, 69, 61 and 65 amino acids for isoforms α (amino acids 177–240), β1 (177–245), β2 (177–237), and β3 (177–241), respectively.

Recombinant HRG and HRG-Toxin Expression. PCR products encoding the HRGs were digested with *Hin*dIII and subcloned into the pFLAG-1 expression vector (International Biotechnologies). After transformation into *E. coli* DH5α, the HRG-coding domains of insert-containing constructs were sequenced (Sequenase version 2.0; USB), and colonies containing cDNA clones coding for each HRG isoform were stored as glycerol stocks. For expression of the ligand toxin, a 1184-bp *Hin*dIII/*Eco*RI cDNA fragment encoding the *Pseudomonas* exotoxin PE40 was subcloned into pFLAG. This initial construct, when expressed in *E. coli*, produces recombinant toxin that was used as a ligand toxin control. The *Hin*dIII cDNA fragment encoding HRGβ2 was then inserted into this toxin construct, which will result in the expression of the HRG-toxin fusion protein (HRGβ2/PE40). The pFLAG-1 prokaryotic expression vector is designed to produce a soluble form of recombinant protein, expressed in the periplasmic space of transformed bacteria, incorporating an 8-amino acid "FLAG" peptide and a 21-amino acid leader sequence (ompA), which allows translocation of the fusion protein to the periplasmic space. The ompA sequence is cleaved during this process. The recombinant FLAG fusion protein can then be purified by affinity chromatography using the M1 anti-FLAG monoclonal antibody.

Cultures of transformed *E. coli* were grown at 37°C with shaking until they reached an absorbance (OD_{600}) of 0.8. Isopropyl-1-thio-β-D-galactopyranoside was then added to 500 μM and cultures incubated for a further 2 h. In an initial experiment to determine the level of expression in each *E. coli* cell fraction, isopropyl-1-thio-β-D-galactopyranoside-induced cultures (100 ml) were divided into three, and cells were initially collected by centrifugation. The whole-cell fraction was isolated by resuspension of one cell pellet in SDS-PAGE sample buffer. The second pellet was used for fractionation of the whole-cell pellet into soluble or insoluble fractions by the addition of 5 ml of extraction buffer A [50 mM Tris-HCl (pH 8.0), 5 mM EDTA, 0.25 mg/ml lysozyme, 50 μg/ml NaN_3] followed by 0.5 ml of extraction buffer B [1.5 M NaCl, 0.1 M $CaCl_2$, 0.1 M $MgCl_2$, 0.02 μg/ml DNase I, 0.2 mM $NaVO_3$, 0.2 mM phenylmethylsulfonyl fluoride, 0.2 mM leupeptin, 0.2 mM aprotinin]. The resulting suspension was centrifuged at 18,000 × *g* for 1 h, giving the soluble and insoluble cell fractions. The insoluble cell fraction was resuspended in SDS-PAGE sample buffer while an aliquot of the soluble fraction was mixed with an equal volume of 2× sample buffer. Finally, the third cell pellet was prepared for osmotic shock by resuspension in 8 ml of OS buffer [0.5 M sucrose, 0.03 M Tris-HCl (pH 8.0), and 1 mM EDTA] and centrifugation at 3500 × *g* for 10 min at

10°C. Cells were resuspended rapidly in 5 ml of ice-cold water to release periplasmic proteins. Following centrifugation at 3500 × *g* for 10 min at 4°C, the supernatant was collected, and a 10-μl aliquot was mixed with an equal volume of SDS-PAGE sample buffer. After electrophoresis and transfer of proteins to nitrocellulose membranes (Bio-Rad), FLAG proteins were detected with the anti-FLAG M2 monoclonal antibody (10 μg/ml) and enhanced chemiluminescence (ECL; Amersham). The M2 antibody detects FLAG proteins consisting of both ompA-cleaved and non-cleaved forms.

Purification of Recombinant Proteins. For each HRG isoform and the ligand toxin, protein expressed in the periplasmic space was isolated and purified by affinity chromatography using the anti-FLAG M1 monoclonal antibody that binds only ompA-cleaved FLAG fusion proteins. This antibody binds to the FLAG peptide only in the presence of calcium; hence, the periplasmic cell fractions were made up to 2 mM $CaCl_2$ in TBS (150 mM NaCl and 50 mM Tris-HCl, pH 7.5) and passed through the column. Bound proteins were eluted in TBS containing 2 mM EDTA and then analyzed for size and purity by Western blot using the M2 anti-FLAG antibody and by Coomassie staining of SDS-PAGE gels.

HRG Stimulation of Tyrosine Phosphorylation of erbB Receptors Expressed in Breast Cancer Cells. MCF-7 human breast cancer cells were grown to near confluence in 6-well tissue culture plates. The cells were then starved for 18 h in medium containing 0.5% FCS. Recombinant HRG (one of the four isoforms) was then added in 100 μl of TBS/0.05% BSA (final concentration, 20 pM to 10 nM) to individual wells. Control wells received vehicle alone. Following a 20-min incubation at 37°C, the medium was removed by aspiration, and cells were collected in 200 μl of lysis buffer [1% Triton X-100, 150 mM NaCl, 10 mM Tris-HCl (pH 7.4), 1 mM EDTA, 0.2 mM Na_3VO_4, 0.2 mM phenylmethylsulfonyl fluoride, 0.2 mM leupeptin, and 0.2 mM aprotinin). Following incubation on ice for 5 min, cellular debris was removed by centrifugation.

To determine the tyrosine phosphorylation levels of erbB2–4 following HRG administration, samples of the resulting cell lysates were separated by SDS-PAGE, transferred to nitrocellulose, and Western blotted using a monoclonal antibody against phosphorylated tyrosine residues (PY20). Detection of bound antibody was by ECL (Amersham).

For immunoprecipitation experiments, ZR-75–1 or MCF-7 breast cancer cells were grown to near confluence in T150 tissue culture flasks. After serum starvation, HRGβ2 (10 nM) was added for 15 min. The cells were then lysed in 1 ml of lysis buffer as described above. Antibodies (1–2 μg) were incubated with lysates (100 μl of lysate for all reactions, except for the detection of erbB4 in PY20 immunoprecipitates, where 250 μl was used) for at least 2 h at 4°C. The immunocomplexes were then collected by incubation with goat antimouse IgG-Sepharose or protein A-Sepharose beads (40 μl) for at least 1 h at 4°C. Sepharose beads were collected by centrifugation and washed three times in cold lysis buffer, resuspended in SDS-PAGE sample buffer, and subjected to SDS-PAGE. After transfer to nitrocellulose, the samples were Western blotted with the desired antibody.

MAP Kinase Assay. MAP kinase p44 (ERK1) was immunoprecipitated from ZR-75–1 cell lysates (250 μl) before and 15 min after HRGβ2 addition and then examined for its ability to phosphorylate an exogenous substrate, MBP (5 μg), in the presence of ATP (20 μM) and [γ-^{32}P]ATP (4 μCi) for 10 min at 30°C. Radioactive bands representing phosphorylated MBP were revealed by autoradiography following SDS-PAGE of the reaction products and band intensities quantified by densitometry. To control for loading differences, aliquots of the ERK1 immunoprecipitates were Western blotted using a different antibody to ERK1 (K23; Santa Cruz Biotechnology).

PI3-kinase Activity Assay. erbB3 protein was immunoprecipitated from 500 μl of cell lysates from HRGβ2-treated or control ZR-75–1 cells using anti-erbB3 monoclonal antibody (2F12). The immunoprecipitates were collected by centrifugation and divided into two aliquots to enable both determination of PI3-kinase activity and analysis of the immunoprecipitates by Western blotting with anti-erbB3 and antiphosphotyrosine antibodies.

PI3-kinase activity was assayed using a method described previously (64). Immunoprecipitates were washed twice with 1% NP40 in PBS, twice in 0.5 M LiCl, 0.1 M Tris (pH 7.6), and twice in TNE buffer [10 mM Tris (pH 7.6), 100 mM NaCl, and 1 mM EDTA]. The reaction was started by the addition of 50 μl of kinase buffer containing 20 mM Tris (pH 7.6), 75 mM NaCl, 10 mM $MgCl_2$, 200 μM adenosine, 20 μM ATP, 10 μCi [γ-^{32}P]ATP, and PI resuspended by sonication in 20 mM HEPES and added to a final concentration of 0.2 mg/ml. After 15 min at room temperature, the reaction was stopped with 100 μl of 1 M HCl, and lipids were extracted with 200 μl of $CHCl_3$:methanol (1:1). The organic phase was recovered, dried under nitrogen gas, and resuspended in 10 μl of $CHCl_3$:methanol (2:1) containing 0.1% HCl. Phospholipid products were separated by TLC on a silica gel 60 plate (Merck), developed in $CHCl_3$:methanol:4 M NH_4OH (9:7:2), and then exposed to autoradiography. Unlabeled phospholipid standards (PI and phosphatidyl inositol 4-phosphate) were included and visualized by exposure to iodine vapor.

Proliferation Assays. Breast cancer cells MCF-7, T-47D (in RPMI 1640 containing 5% FCS), ovarian cancer cells (in RPMI 1640 containing 5% FCS and 100 units/ml of insulin), and normal breast epithelial cells 184B5 (in mammary epithelial growth medium) were dispensed into individual wells of 96-well plates at an initial concentration of 10^3 cells/well in 50 μl of growth media, 3 days before (day −3) the addition of recombinant HRGβ2, HRGβ2/PE40, or PE40. At day 0, recombinant proteins were added in 50 μl of growth media to final concentrations ranging from 0.5 pM to 5 nM. Controls received either an equivalent volume of vehicle (0.5 μl of 0.1% BSA) in 50 μl of growth media or growth media alone. Three plates of identical treatments were prepared, and one plate was analyzed for cell growth on each of days 3, 5, and 7 after drug addition. Relative cell numbers (6 wells for each treatment) were measured indirectly using a nonradioactive cell proliferation assay (Promega) by absorbance readings (A_{595}-A_{630}) using a Dynatech MR7000 Spectrophotometer.

Flow Cytometry. T-47D human breast cancer cells were maintained under serum-free conditions in T25 flasks, and the effects of 5% FCS, 10 μg/ml insulin, and 5 nM HRGβ2 on cell cycle progression were determined as described previously (42).

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